ADVANCES IN YEAST AND MOLD MONODRUG AND COMBINATION DRUG ANTIFUNGAL SUSCEPTIBILITY TESTING

by

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ABSTRACT

Advances were made in both yeast and mold rapid susceptibility assay (RSA) testing. The yeast RSA was modified to facilitate amphotericin B (AMB), itraconazole (ITC), and voriconazole (VRC) testing of *Aspergillus fumigatus*, *A. terreus,* and *A. flavus* clinical isolates. 16 h mold RSA AMB, ITC, and VRC RSA minimum inhibitory concentration (MIC) values were equal to or within a single, two-fold dilution of MICs obtained in 48 h with the National Committee for Clinical Laboratory Standards (NCCLS) M38-A assay and in 24 or 48 h with the mold Etest. Preliminary testing with *A. sydowii*, *Scedosporium angiospermum*, and *Fusarium oxysporum* suggests that the mold RSA would also be a suitable AMB and ITC susceptibility testing format for these opportunistic filamentous fungi. The AMB, ITC, and VRC susceptibilities of *A. fumigatus* conidia and hyphae were compared by modifying the mold RSA, with conidia and hyphae demonstrating similar susceptibilities to drug. The yeast RSA was validated by testing clinical control strains that were obtained from patients with known clinical outcome. Yeast RSA conditions were also optimized to facilitate FLC testing of *C. glabrata* isolates.

The effects of concurrent and sequential amphotericin B, itraconazole, and voriconazole two-drug combinations on the conidial and hyphal inocula of *A. fumigatus* were determined using a checkerboard testing format, with drug interaction effects interpreted by calculating fractional inhibitory concentration indices. Our interpretations of the in vitro effects of concurrent and sequential antifungal combinations on *A. fumigatus* closely matched the reported outcomes of invasive aspergillosis patients treated with concurrent and sequential antifungal therapies. Importantly, both concurrent and sequential antifungal combinations had differential effects on conidial and hyphal inocula, suggesting that all combination testing be performed with hyphal inocula.

INTRODUCTION

Epidemiology of Medically Significant Mycoses

Fungal pathogens can be divided into two categories: 1) primary pathogens, that cause disease in immunocompetent or immunocompromised individuals; and 2) opportunistic pathogens, which primarily cause disease in immunocompromised individuals (1). Five fungi are classified as primary pathogens: *Coccidioides immitis*, *Paracoccidioides brasiliensis*, *Blastomyces dermatitidis*, *Penicillium marneffei*, and *Histoplasma capsulatum* spp. (2, 3). Susceptibility to these pathogens has traditionally been correlated with geographic location (4). While mycoses caused by primary pathogens are medically significant and worthy of a more detailed discussion, they are not the focus of this thesis and will therefore not be discussed in greater detail. This thesis will instead focus on the opportunistic pathogens, namely *Aspergillus* and *Candida* spp.

Opportunistic fungal infections can be caused by an almost unlimited number of yeast and filamentous fungal pathogens (5), with the most prevalent etiologic agents being: *Aspergillus fumigatus*, *A. flavus*, *A. niger*, *A. terreus*, *Candida albicans*, *C. glabrata*, *C. tropicalis*, *C. krusei*, *C. parapsilosis*, *Cryptococcus neoformans*, hyalohyphomycetes, phaeohyphomycetes, and zygomycetes. The incidence rates of infection caused by these pathogens have greatly increased since the early 1980's when both the onset of AIDS and advancements made in the fields of chemotherapy and organ transplantation resulted in increased numbers of immunocompromised patients (6-10).

Table 1. Abbreviations used in Chapter 1.

Term	Abbreviation
Allergic Bronchopulmonary Aspergillosis	ABPA
Amphotericin B	AMB
5-Fluorocytosine	5-FC
Fluconazole	FLC
Itraconazole	ITC
Amphotericin B Colloidal Dispersion	ABCD
Amphotericin B Lipid Complex	ABLC
Caspofungin Acetate	CAS
Voriconazole	VRC
P450 14α-demethylase	$P450_{14\alpha dm}$
Ravuconazole	RCZ
Posaconazole	PCZ
Antifungal Susceptibility Testing	AST
Antifungal Resistance Testing	ART
National Committee for Clinical Laboratory Standards	NCCLS
Polymerase Chain Reaction	PCR
Minimum Inhibitory Concentration	MIC
Nikkomycin Z	NKZ
Colony Forming Units	CFU
American Type Culture Collection	ATCC
Optical Density	OD
Elipsometer Test	Etest
Rapid Susceptibility Assay	RSA
Invasive Aspergillosis	IA
Fractional Inhibitory Concentration	FIC
Amphotericin B and Itraconazole	AMB+ITC
Amphotericin B and Voriconazole	AMB+VRC
Itraconazole and Voriconazole	ITC+VRC
Amphotericin B followed by Itraconazole	AMB→ITC
Amphotericin B followed by Voriconazole	AMB→VRC
Itraconazole followed by Amphotericin B	ITC→AMB
Itraconazole followed by Voriconazole	ITC→VRC
Voriconazole followed by Amphotericin B	VRC→AMB
Voriconazole followed by Itraconazole	VRC→ITC

Table 2. Relative proportion of opportunistic nosocomial infections, by pathogens, 1980 to 1990[a]

Opportunistic Pathogen	Estimated Percentage
Candida albicans	61
Non-*albicans Candida* spp.	27
C. glabrata	
C. parapsilosis	
C. tropicalis	
C. krusei	
Aspergillus spp.	1
Other	11
Yeasts	
Malassezia sp.	
Trichosporon sp.	
Zygomycetes	
Mucor sp.	
Rhizopus sp.	
Hyalohyphomycetes	
Fusarium sp.	
Acremonium sp.	
Phaeohyalohyphomycetes	
Alternaria sp.	
Bipolaris sp.	
Curvularia sp.	

[a] Adapted from reference (3). Data from references 4 and 9.

The relative proportions of hospital-associated, or nosocomial, fungal infection by fungal pathogen between 1980 and 1990 are shown in Table 2 (3, 4, 9). *C. albicans* and non-*C. albicans* species of *C. glabrata*, *C. parapsilosis*, *C. tropicalis*, and *C. krusei* are clearly the most prevalent etiologic agents of opportunistic infections. More recent surveillance data have shown that *Candida* spp. continue to account for the greatest percentage of nosocomial fungal infections (9, 11). Although *Aspergillus* spp. can cause

devastating infections in specialized immunocompromised populations, the overall percentage of nosocomial infections attributable to *Aspergillus* has remained low, at approximately 1%. Of increasing importance are the nosocomial infections caused by a rising number of "other" fungi, with the most prevalent pathogens including non-*C. albicans* yeasts, such as *Malassezia* and *Trichosporon* spp., and filamentous fungi such as zygomycetes, hyalohyphomycetes, and phaeohyphomycetes (3).

Although morbidity rates have continued to increase since the 1980's, trends in mortality rates appear to be pathogen-specific. Mortality due to *Candida* spp. increased during the 1980's, but began to decrease in the 1990's. This decline in mortality rates has been attributed to improvements in both early detection and management of invasive infection. In contrast, mortality due to *Aspergillus* spp. has continued to increase since the 1980's, now surpassing the mortality rate of *Candida* infections (12).

While over 100 species of *Candida* have been identified, only those listed in Table 1 are significant pathogens of nosocomial infections. *Candida* species are

Table 3. Candida species distribution in sentinel and population-based candidiasis surveillance programs in the USA

Years	References	No. Isolates	% of total isolates					
			C. albicans	*C. glabrata*	*C. parapsilosis*	*C. tropicalis*	*C. krusei*	Other
1987-1988	5	137	47	14	16	20	0	3
1989-1993	15	232	56	13	10	17	2	2
1990-1994	16	427	52	16	11	15	4	2
1992-1993	17	394	46	15	21	12	3	3
1992-1993	18	428	51	12	22	8	-	7
1995-1996	11	379	52	20	8	11	5	4
1995-1998	19	934	53	20	10	12	3	2
1997-2000	20	2047	54	16	15	10	2	3

ubiquitous colonizers of humans and can often be isolated from the gastrointestinal tract,

Table 4. Candida species distribution in sentinel and population-based candidiasis surveillance programs outside the USA

Years	References	No. Isolates	Location	% of total isolates					
				C. albicans	*C. glabrata*	*C. parapsilosis*	*C. tropicalis*	*C. krusei*	Other
1980-199	21	177	Iceland	64	12	10	6	<1	8
1991-1996	22	576	Norway	66	13	8	6	2	6
1992-1994	23	249	multiple	49	10	11	11	9	10
1992-1994	24	367	Canada	71	9	8	7	1	4
1994-1994	25	293	Israel	53	7	12	11	1	15
1996-1998	26	442	Belgium	54	15	12	9	3	6
1996-2001	27	218	Spain	42	12	22	16	6	2
1997-1998	28	579	Phillipines	50	6	17	15	2	10
1997-1998	29	20900	multiple	69	10	4	4	2	12

vagina, urethra, or skin (13). These pathogens cause a wide spectrum of diseases that affect the gastrointestinal tract, genitalia, skin, nails, and deep tissues. *Candida* infections in deep tissues are usually the result of hematogenous spread of *Candida* from an endogenous, or less frequently, an exogenous site (13). Development of candidiasis is dependent on the underlying host factors of the immunocompromised patient, with specific predisposing factors putting the patient at risk for developing particular manifestations of disease. The attributable mortality rate due to candidiasis is approximately 38% (14), with infections most frequently occurring in medical and surgical hospital services (3, 9). While *C. albicans* has consistently caused the highest proportion of *Candida* spp. nosocomial fungal infections since the 1980's, the distribution of infections caused by non-*C. albicans* species may have shifted during this time. The majority of recent sentinel and population-based surveillance studies conducted in the United States show that *C. glabrata* is beginning to replace *C. tropicalis* as the second most prevalent nosocomial *Candida* pathogen (Table 3) (5,11,15-20).

Surveillance studies conducted outside the Unites States are less clear, with the increased prevalence of *C. glabrata* appearing to be site specific (Table 4) (21-29). The changing distributions of non-*C. albicans* species has also been found to be age specific (17, 20, 30, 31), with data of one representative study (20) shown in Table 5. *C. albicans* and *C. parapsilosis* are predominant in neonatal and pediatric infections while *C. albicans* and *C. glabrata* infections predominate in adult infections. Within the adult population, the proportion of *C. albicans* infections decrease while the proportion of *C. glabrata* infections increase with increasing patient age (20, 32).

Table 5. *Candida* species prevalence distribution by age group[a]

Age Group	% Total					
	C. albicans	*C. glabrata*	*C. parapsilosis*	*C. tropicalis*	*C. krusei*	Other spp.
≤1	60	3	24	7	0	5
2-15	55	3	21	10	4	7
16-64	55	17	12	11	2	3
≥64	50	23	12	10	2	3

[a] Reference 20

While over 200 species of *Aspergillus* have been identified, only a limited number have been associated with disease (Table 6) (33, 34). *Aspergillus* spp. can cause a variety of manifestations including pulmonary aspergilloma, allergic bronchopulmonary aspergillosis (ABPA), and invasive aspergillosis (20). *A. fumigatus* is the most commonly isolated species in invasive and noninvasive aspergillosis (35). *A. flavus* is the second most common species isolated in invasive aspergillosis and nasal sinus lesions (35, 36). *A. niger* is the second most common agent of pulmonary aspergilloma and third

most common agent of invasive pulmonary aspergillosis (35). *A. terreus* is the fourth most common agent of invasive aspergillosis (35). Although the numbers of nosocomial infections caused by *Aspergillus* are small compared to *Candida*, the incidence of invasive *Aspergillus* infections can be high in individuals who are immunocompromised due to burn injury, malignancy, leukemia, solid-organ transplantation, and bone marrow transplantation (3, 37-40). Bone marrow patients are especially at risk for developing aspergillosis, with incidence rates ranging from 10 to 30% (3, 37, 41). Although difficult to determine due to the high mortality rate of susceptible patients, the attributable mortality of invasive aspergillosis has been estimated to range from 13 to 95%, with the highest rates seen in patients with aplastic anemia and bone marrow transplantation (3, 37, 41). Despite recent advances in antifungal development, the survival rate with antifungal therapy still remains low at 34 to 50 % (42, 43).

Table 6. Agents of aspergillosis[a]

A. fumigatus	*A. fischeri*	*A. sydowii*
A. flavus	*A. flavipes*	*A. ustus*
A. niger	*A. nidulans*	*A. versicolor*
A. terreus	*A. niveus*	
A. amstelodami	*A. ochraceus*	
A. candidus	*A. oryzae*	
A. carneus	*A. parasiticus*	
A. clavatus	*A. repens*	
A. conicus	*A, restrictus*	
A. deflectus	*A. ruber*	

[a] Reference {339}.

Antifungal Drugs and Therapy

Evolution of Antifungal Drug Development and Therapy

Until the 1950's, the only antifungal therapy available was the oral administration of a saturated solution of potassium iodide for cutaneous sporotrichosis (44). A broader spectrum antifungal drug was not discovered until 1951, when Brown and Hazen isolated the polyenes nystatin, hamycin, and amphotericin B (AMB) from the actinomycete *Streptomyces nodosus* (45,46). One of the earliest successful applications of AMB therapy was the treatment of coccidioidal meningitis, a universally fatal manifestation of coccidioidomycosis (47, 48). Once demonstrated with coccidioidomycosis, AMB was soon successfully tested and used as a therapy for aspergillosis, cryptococcosis, candidiasis, and other fungal infections (47, 49-51). Unfortunately, AMB's greatest limitation is its induction of nephrotoxicity in patients. Treatment with AMB usually results in some degree of renal dysfunction, with severity correlating to the total AMB dose (49, 52). Despite this limitation, AMB continues to be the mainstay of management for many fungal infections.

5-Fluorocytosine (5-FC) was next developed in the late 1950's, originally as an anti-tumor agent (53). Although 5-FC has demonstrated antifungal activity in vitro and in vivo, its use as an antifungal monotherapy has been limited due to a spectrum of activity limited to *Candida* and *Cryptococcus* (54), bone marrow toxicity (55), and the rapid development of resistance in vivo (56, 57). While 5-FC is no longer used as a monotherapy, clinical trials and case studies have demonstrated the effectiveness of 5-FC

in combination with AMB or fluconazole (FLC) for treating cryptococcal meningitis (58-64) or chromoblastomycosis (65, 66).

During the 1970's, a series of antifungals, termed imidazoles, were released. These drugs demonstrated a greater spectrum of activity, with both topical and systemic applications. Clotrimazole was well tolerated during treatment but was rapidly metabolized due to its induction of hepatic P450 enzymes (67). Unlike clotrimazole, miconazole was not rapidly metabolized, but due to its poor solubility in water and adverse effects, was limited in use (68). These systemic complications have now limited both clotrimazole and miconazole to topical use. The third and most successful imidazole was ketoconazole, an orally administered, relatively non-toxic drug with a broad spectrum of activity that included *Candida* (69), *Histoplasma* (70, 71), *Paracoccidioides* (70), *Blastomyces* (71, 72), *Coccidioides* (73), *Sporothrix* (74), dermatophytes, and dematiaceous fungi (75). Despite the success of ketoconazole in treating these infections, fungi such as *Aspergillus*, *Fusarium*, and zygomycetes were intrinsically resistant, and amphotericin B remained the only treatment option (76).

During the 1980's, the incidence of mycoses doubled due to an increasing population of immunocompromised patients resulting from the AIDS epidemic and advances being made in chemotherapy and bone marrow and solid organ transplantation (9). The pharmaceutical industry soon responded, with multiple companies initiating research programs to develop new antifungal drugs. By the 1990's, both fluconazole (FLC) and itraconazole (ITC), collectively called triazoles, were available for treating a wide spectrum of mycoses. Due to its low toxicity and therapeutic efficacy equal to

AMB, FLC has become the standard antifungal therapy for uncomplicated candidiasis in non-neutropenic and neutropenic patients, although AMB is still recommended as first-line therapy in clinically unstable cases of candidiasis. FLC therapy is well tolerated in most patients, but prolonged therapy and extensive prophylactic use of FLC has resulted in acquired FLC resistance in up to 10% of strains isolated from certain candidiasis patient groups (77, 78). In addition, FLC is ineffective in treating *Aspergillus* spp. and *C. krusei* infections, as both pathogens are intrinsically resistant. Originally licensed in 1991, ITC is now available in both oral and i.v. formulations. ITC has a broad antifungal spectrum, with in vitro and in vivo activity against *Aspergillus*, *Blastomyces*, *Candida*, *Coccidioides*, *Cryptococcus*, *Histoplasma*, *Paracoccidioides*, *Scedosporium*, *Sporothrix*, dermatophytic, and dematiaceous spp. ITC is ineffective, however, against *Fusarium*, *Scedosporium*, and zygomycetes spp. Traditionally, the greatest limitations of ITC use have been poor gastric adsorption and complex drug interactions. However, the development of an ITC-β-hydroxydextrin formulation has resulted in almost complete gastric adsorption. An i.v. formula has also been developed, eliminating any adsorption issues.

The issue of AMB induced nephrotoxicity was recently addressed by the development of three liposomal forms of AMB, which are less toxic than AMB, allowing administration at higher doses (12, 79). Two of these lipid formulations, amphotericin B colloidal dispersion (ABCD) and amphotericin B lipid complex (ABLC), have been shown to improve survival in aspergillosis patients who were no longer responding or intolerant to AMB (80, 81). Although these lipid formulations result in therapeutic

efficacy equal to AMB in first-line aspergillosis therapy (82), increased doses are not associated with improved outcome (83). Unfortunately, the greatest limitation of using these drugs has been the cost, with newer formulations of AMB costing approximately 40 to 50 times more than AMB (84).

Caspofungin acetate (CAS), an echinocandin, was approved by the U.S. Food and Drug Administration (FDA) in 2001 for the treatment of *Aspergillus* infection refractory to conventional therapy (85). Randomized clinical trials have demonstrated that CAS has equal efficacy but less toxicity than AMB in treating esophageal and oropharyngeal candidiasis (86, 87). Although CAS has been shown to be effect against both *Aspergillus* and *Candida* spp., both *Cryptococcus neoformans* and zygomycetes are intrinsically resistant (88).

Voriconazole (VRC), a second generation triazole approved by the FDA in 2002, has been shown to be effective as either a rescue (89) or first-line therapy for invasive aspergillosis (43, 90). Importantly, Herbrecht et al. showed that VRC therapy resulted in better rates of response and survival compared to AMB therapy (43). Although VRC therapy causes photopsia, a transient enhanced light perception or blindness, in up to 45% of patients, the effect is temporary and does not require discontinuation of therapy (43). VRC has also been approved for treating serious fungal infections caused by *Scedosporium angiospermum* and *Fusarium* that are intolerant or refractory to other therapy (91).

Other drugs such as posaconazole (PCZ, derivative of ITC), ravuconazole (RCZ, derivative of FLC), nikkomycin Z, micafungin, liposomal nystatin, and anidulafungin are

currently in development or clinical trials and will hopefully be released in the next few years.

Antifungal Drug Classes and Targets in the Fungal Cell

As both mammalian and fungal cells are eukaryotic, the greatest challenge in antifungal drug development has been identification of fungal cell specific targets and restriction of the antifungal drug's inhibitory action(s) to fungal and not mammalian cells. Although there are several major fungal targets, we will limit our discussion to antifungal targeting of fungal cell wall synthesis and ergosterol production.

Cell Wall Synthesis. Components of the cell wall of fungi are ideal antifungal drug targets as they are both essential to the fungal cell and absent from mammalian cells (84). The most notable classes of antifungal drugs targeting components in the cell wall include the echinocandins and nikkomycins. Echinocandins, which include CAS, micafungin, and anidulafungin (74), are noncompetitive inhibitors of the cell membrane enzyme β-(1,3)-glucan synthase, which catalyzes the polymerization of uridine diphosphate-glucose into β-(1,3)-glucan, a structural component of the fungal cell wall partially responsible for maintaining integrity and rigidity (84) (Fig. 1). Inhibition of β-(1,3)-glucan synthesis leads to a weakened cell wall, increased osmotic pressure, and ultimately lysis. Nikkomycin Z is structurally similar to a substrate precursor for chitin and acts as a competitive inhibitor of chitin synthase enzymes (Fig. 1), leading to osmotic swelling, chaining, and inhibition of septation (53).

Cell Membrane Function. The polyenes (AMB, nystatin) have been proposed to act on a number of fungal cell targets, all involving the cellular membrane. The most documented target of AMB is ergosterol (Fig. 1), the fungal cell membrane sterol

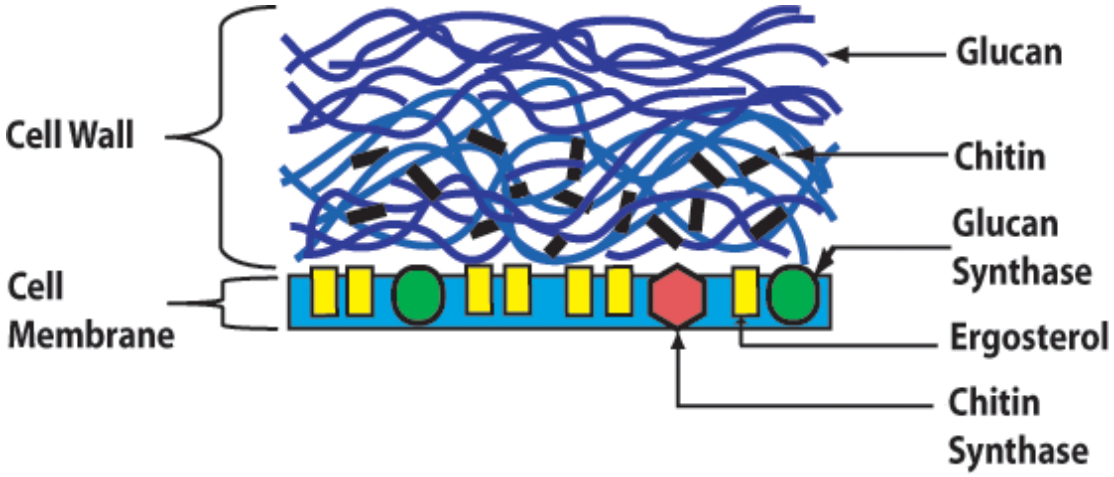


Figure 1. Representation of select fungal cell wall and membrane components.

equivalent to cholesterol in mammalian cells. This "sterol hypothesis" is supported by three lines of evidence (92): 1) the presence of ergosterol in natural or artificial membranes makes them sensitive to AMB; 2) free sterols antagonize the effects of AMB on *Saccharomyces cerevisiae* and *C. albicans*; and 3) several physical-chemical methods demonstrate an AMB-sterol interaction in water or water-methanol mixtures. It is interesting, however, that AMB can also induce sensitivity in sterol-free model membranes, suggesting that sterols may not be the only fungal cell membrane target (92). AMB specificity for fungal ergosterol versus mammalian cholesterol was demonstrated by Readio and Bittman, with AMB binding 10-fold more tightly to unilamellar vesicles composed of ergosterol than those containing cholesterol (93). After binding to

ergosterol via its two hydrophobic chains (77), AMB induces formation of minute pores, causing leakage of K+ and influx of Na+ which results in an ionic imbalance (92, 94, 95). This permeability has been hypothesized to cause leakage of additional intracellular components, resulting in the subsequent death of the fungal cell (96).

AMB has also been proposed to have both immunostimulatory and immunosuppressive effects on the host. AMB has an immunostimulatory effect on macrophages by increasing oxidative bursts (97) and killing of *A. fumigatus* conidia (98). AMB causes immunosuppression by disrupting the cell membranes of polymorphonuclear lymphocytes (PMN), thereby preventing the PMN's oxidative burst (99). Another immunosuppressive property of AMB is that it can induce lipid peroxidation of the mammalian cell membranes, causing fragility in the membrane that may lead to increased permeability (100, 101).

Ergosterol Synthesis The ergosterol synthesis pathway and proposed azole target are shown in Figure 2. The primary mode of azole action has been demonstrated to be inhibition of ergosterol biosynthesis (102) through the selective inhibition of the enzyme, P450 14α-demethylase ($P450_{14\alpha dm}$) following the stochiometric interaction of the N-3 (imidazole) or the N-4 (triazoles) substituents of the azole ring with the heme of $P450_{14\alpha dm}$ (103, 104). This interaction prevents $P450_{14\alpha dm}$'s ability to remove the C-14 methyl group from lanosterol, resulting in the accumulation of 14α methylsterols in the cell membrane instead of ergosterol (53). While physiochemical similarities exist between ergosterol and many of its precursors, few of these intermediates are able to replace ergosterol as a fluidity regulator within the cell membrane (103, 105, 106).

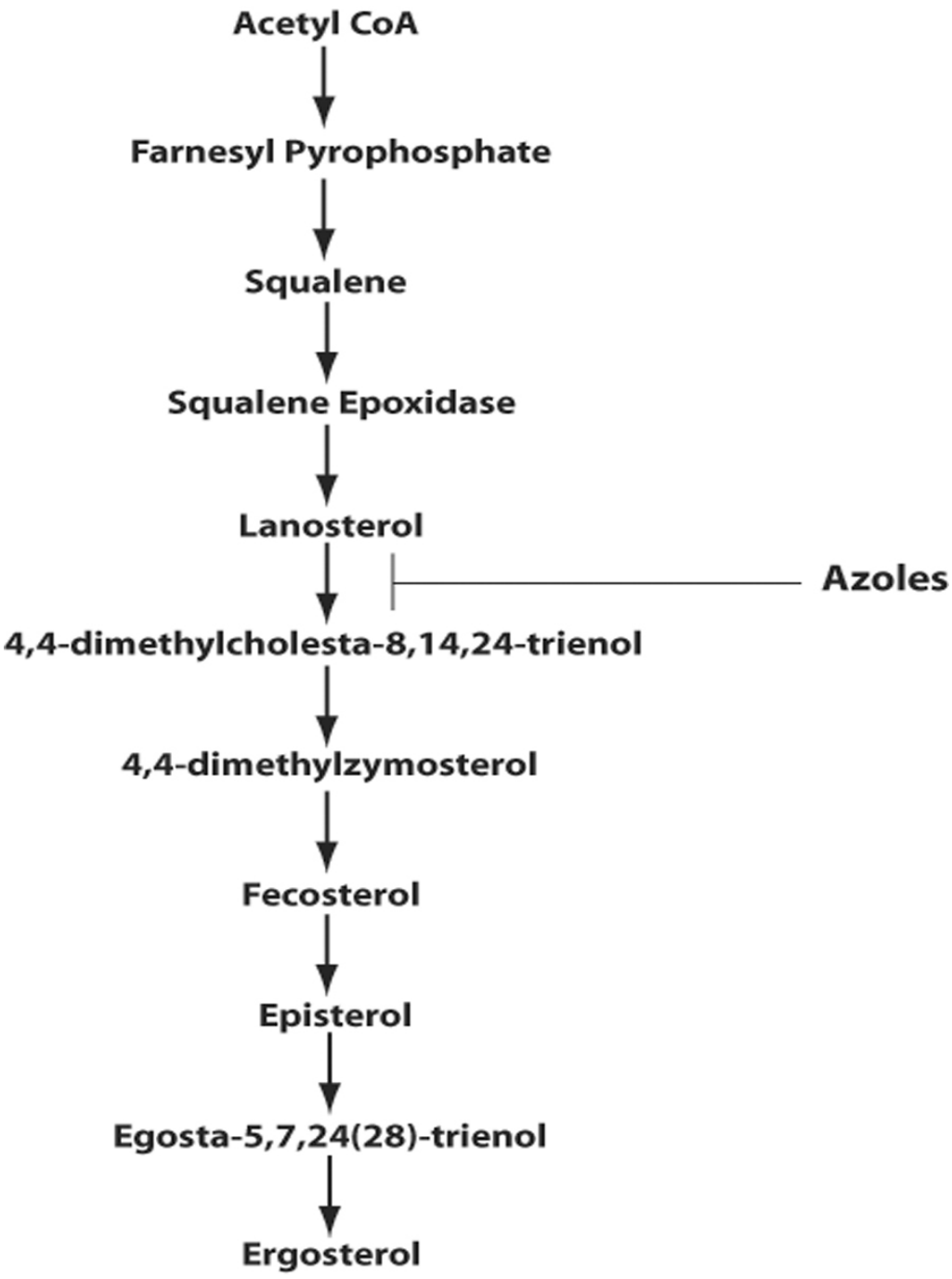


Figure 2. Ergosterol synthesis pathway and target of azoles.

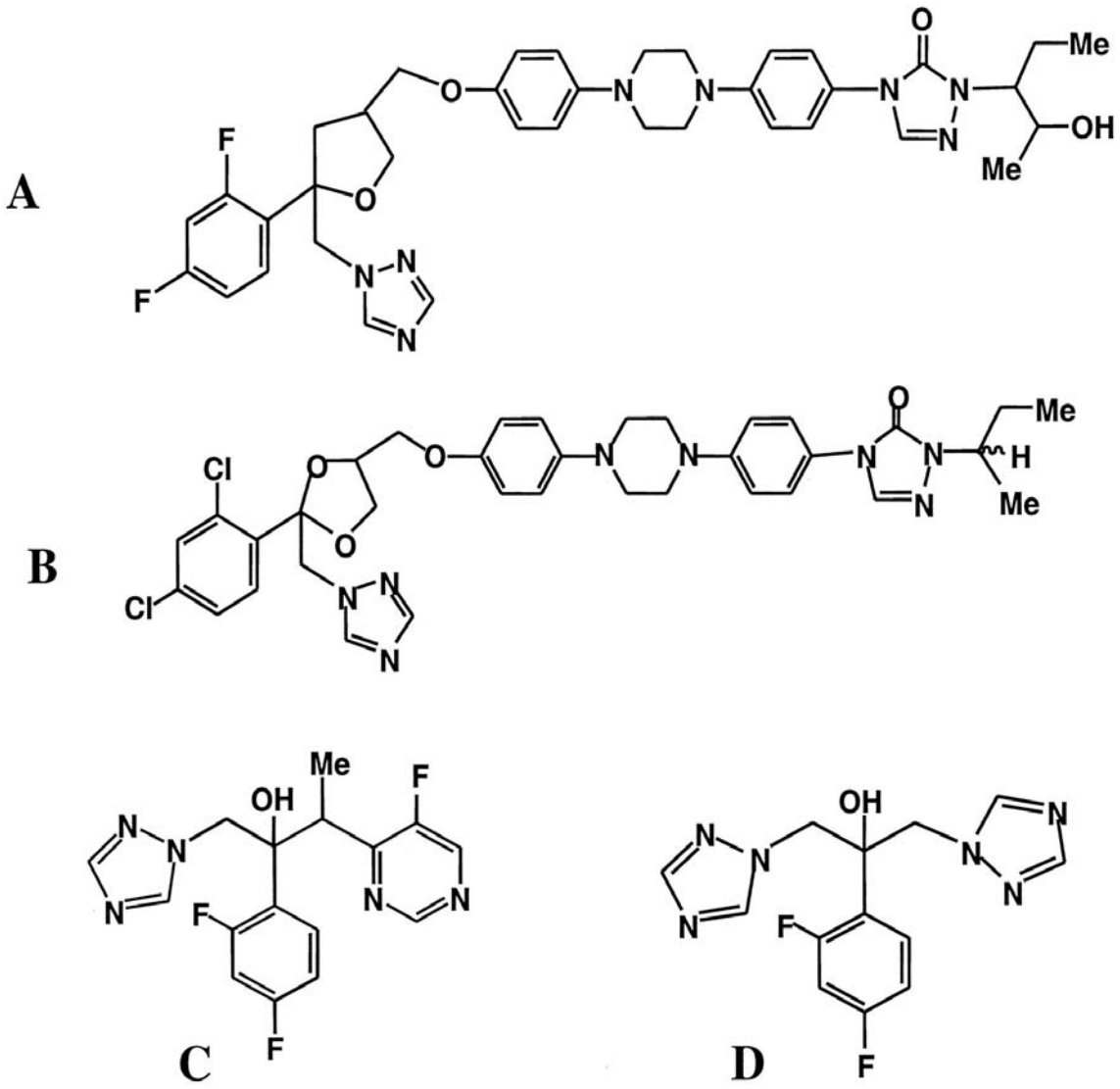


Figure 3. Structures of PCZ (A), ITC (B), VRC (C), and FLC (D).

A large body of *A. fumigatus* literature has indirectly implied that VRC and RCZ interact differently with $P450_{14\alpha dm}$ than ITC and its derivative PCZ (107-110). This was recently confirmed by Xiao et al., who constructed a homology model of the $P450_{14\alpha dm}$ (CYP51A) enzyme of *A. fumigatus* based on the X-ray crystal structure of CYP51 from

Mycobacterium tuberculosis (109). According to the model, triazoles with (Fig. 3A, B) and without (Fig. 3C, D) long chains enter CYP51A via a channel and bind to both heme and nearby channel proteins at the bottom. Interestingly, VRC and PCZ having similar interactions with the channel proteins near the heme. Although VRC occupies a small portion of the channel, the long chains of both ITC and PCZ occupy its full length, forming numerous hydrophobic interactions with channel proteins that are not observed with VRC. The only marked difference between ITC and PCZ binding is that the two chlorine atoms on the steric ring of ITC may contribute to steric crowding near the drug channel entry. Although Xiao and others have developed *C. albicans* homolog models (111, 112), this is the first *A. fumigatus* homolog model, whose success relies greatly on its ability to explain the inconsistency of triazole cross-resistance between ITC/PCZ and VRC in *A. fumigatus* clinical isolates (discussed below).

Amphotericin B and Azole Resistance in *Aspergillus* spp.

The term "resistance" is used to describe the insensitivity of a fungus to an antifungal drug in vitro or in vivo. In contrast, clinical failure describes the failure of an antifungal therapy to result in a clinical response in vivo. Clinical failure can be due to antifungal resistance, but may also be due to host or pharmacodynamic causes such as impaired immune status, poor availability of drug, and accelerated metabolism of the antifungal drug. Resistance can be further classified into three categories: primary or intrinsic resistance, when all isolates of one species are resistant to a particular antifungal drug; secondary or acquired resistance, when a fungus becomes resistant after exposure

to antifungal drug; and clinical resistance, when failure of therapy is due to host factors unrelated to in vitro resistance (113).

Amphotericin B Resistance. The proposed mechanism(s) of AMB resistance among fungi are varied and often conflicting, adding support to the argument that AMB has more than one target and/or that host factors may influence the efficacy of AMB in vivo. AMB resistance is either acquired during prolonged AMB therapy or intrinsic, depending on the fungal species. As ergosterol is proposed to be the primary target of AMB, many investigators have hypothesized that AMB resistance is associated with either increased or decreased ergosterol content in the cellular membrane. However, these alterations in ergosterol content have not consistently correlated with AMB resistance. For example, ergosterol levels in the cell membrane of *A. terreus*, a fungus intrinsically resistant to AMB in vivo, are either decreased (114) or no different than (114-116) ergosterol levels in AMB-sensitive strains of *A. fumigatus*. Ergosterol levels in the membranes of other intrinsically resistant fungi such as *Scedosporium prolificans* and *Fusarium* spp. should be determined to help clarify the relationship between ergosterol content in fungal membranes and AMB sensitivity.

As AMB must first cross the cell wall to reach ergosterol in the cell membrane, an alternative hypothesis for AMB resistance is that constituent(s) in the cell wall, namely glucan and/or chitin, interfere with AMB-ergosterol binding by interacting with or altering the permeability of AMB. Using AMB-sensitive and resistant strains of *A. flavus*, Seo et al. found that spheroplasts derived from resistant mycelia were as susceptible to AMB as wild-type hyphae, suggesting that alterations in the cell wall lead

to AMB resistance (117). The role of glucan/chitin-AMB interactions in AMB resistance has been investigated in greater detail with AMB-sensitive *C. albicans* and AMB-sensitive and –resistant *Schizosaccharomyces* sp. and *Kluyveromyces* sp. The increased AMB sensitivity of yeast spheroplasts, which lack cell walls, also supports the role of the cell wall as a chemical barrier around the fungal cell (116). However, Bahmed et al. found that chitin content was proportional to AMB susceptibility, with increased levels of chitin, induced by addition of chitin synthase activator α-factor, correlating with increased AMB sensitivity, and decreased levels of chitin, caused by addition of the nikkomycin Z, correlating with decreased AMB sensitivity (116). In addition to AMB-chitin interactions, Gale has demonstrated that phenotypic AMB resistance in *C. albicans* can be mediated by alterations in β-(1,3) glucan (118). Although these results suggest a role for the cell wall in determining AMB sensitivity, the mechanism(s) of glucan- or chitin-AMB interactions in AMB cell wall penetration of *C. albicans* have not been elucidated.

Azole Resistance Resistance mechanisms observed in ITC, VRC, or PCZ-resistant laboratory and clinical strains of *Aspergillus* include mutations of the azole target, cyp51A (108, 110, 119) and reduction of intracellular drug levels (120) by efflux pumps AfuMDR3 and AfuMDR4 (121), Plasmid-mediated overexpression of the P-450 14α-sterol demethylase target, cyp51A has also been shown to increase azole resistance (122), but there have been no in vitro or in vivo reports crediting cyp51A overexpression as the cause for resistance.

Table 7. Correlation between amino acid substitutions in codon 54 of cyp51A and decreases in azole susceptibility in clinical isolates and spontaneous mutants of *A. fumigatus*

A. fumigatus strain	Substitution in CYP51A	NCCLS M38-A MIC (μg/ml)[a]			References
		VRC	ITC	PCZ	
ND158	None	0.25	0.12	0.03	108, 109
AF-71	None	1	1	0.25	107
MS6	G54R	0.12	**>16**	1	108,109
ND223	G54R	0.12	**>16**	1	108
AF-72	G54E	1	**>8**	0.5	107, 110
R4-1	G54E	0.25	**>16**	1	108, 109
R7-1	G54W	0.25	**>16**	**>8**	108
ND202	G54W	0.12	**>16**	**>8**	108
F10	G138R	**16**	2	0.25	109
F33	G138S	**8**	0.5	0.25	109

[a] MIC values indicating in vitro resistance are printed in bold.

Unlike *C. albicans*, where FLC-resistant strains consistently exhibit cross-resistance with ITC and VRC, azole cross-resistance in *Aspergillus* is more complicated (Table 7). ITC-resistant *A. fumigatus* strains are not consistently resistant to either VRC or PCZ (107-110, 123) while PCZ-resistant *A. fumigatus* strains have thus far been consistently resistant to ITC but not to VRC (108). This pattern of resistance may be explained by again returning to the *A. fumigatus* CYP51A homolog model developed by Xiao et al (109). The cyp51A gene of ITC and PCZ, but not VRC, -resistant *A. fumigatus* strains has been shown to have a point mutation in codon 54, changing the amino acid from glycine, a small hydrophophic amino acid, to valine, glutamate, or arginine in ITC-resistant strains and to tryptophan in PCZ-resistant strains. According to Xiao's

homology model, codon 54 is located at the entrance of the CYP51A azole docking channel. A substitution of glycine with a larger amino acid could therefore interfere with entry of the long side chains of ITC and PCZ into the channel without affecting VRC entry. As the two chlorine atoms on the phenyl ring of ITC already cause steric crowding at the channel entrance in the absence of a substitution of codon 54, the substitution of a small (valine) or medium (glutamate) amino acid would be sufficient to inhibit ITC channel entry. In contrast, PCZ does not have Cl atoms on its phenyl ring, implying that the glycine must be substituted with a larger amino acid, such as tryptophan, to prevent PCZ channel entry. In contrast, VRC resistance is dependent on amino acid substitution occurring at the base of the docking channel, near the heme binding site. While these substitutions would also affect ITC and PCZ binding, Xiao proposes that the high-affinity binding of ITC and VRC long chains to hydrophobic amino acid along the length of the channel wall compensates for these substitutions, thus preventing ITC and VRC resistance.

Monodrug Antifungal Susceptibility Testing

In vitro antifungal susceptibility testing is used to determine the susceptibility of a clinical fungal isolate to antifungal drug in vitro. The ultimate goal of this testing is to use the resulting in vitro minimum inhibitory concentration (MIC) of antifungal drug as a predictor of antifungal efficacy in the patient (i.e. in vivo). In addition to its clinical use, susceptibility testing can also be used in both drug development, as a screening tool, and

epidemiological studies, to determine and/or monitor patterns of susceptibility or resistance in defined populations.

Until new antifungal agents were introduced in the 1980's, antifungal susceptibility testing was not considered necessary as there was only one therapeutic option available for most mycoses. Susceptibility testing has since become necessary due to increasing numbers of available antifungal drugs, antifungal resistance, numbers of opportunistic fungal pathogens, and incidence of fungal infections (124).

There has been some debate as to whether antimicrobial susceptibility testing is the best testing format for determining the effect of antifungal drugs on fungi in vitro. While susceptibility testing has been used traditionally for both bacteria and fungi to determine the minimum inhibitory concentration (MIC) of drug, it may not detect low level resistance or resistance mechanisms expressed under certain conditions (i.e. in vivo). The alternative to susceptibility testing is antimicrobial resistance testing, a testing format designed to detect phenotypic resistance. The ultimate goal of both testing formats is to extrapolate the results to predict clinical efficacy in the patient. In other words, a successful in vitro test will result in an in vitro-in vivo correlation. The consensus from studies comparing the vitro-in vivo correlation success of bacterial susceptibility and resistance testing is that resistance carries a stronger prediction of failure while susceptibility carries no guarantee of success (125). As previously discussed, this is not surprising as clinical success often depends on the appropriateness of therapy as well as host and/or infection factors (125). Despite the apparent advantages of resistance testing, the majority of antifungal susceptibility tests have been developed in the susceptibility

testing format. Antifungal resistance tests are starting to be developed, however, as molecular mechanisms of resistance, i.e. phenotypic resistance, can now be determined by polymerase chain reaction (PCR) and real-time PCR based techniques (108, 110, 121). Regardless of the development of these resistance tests, investigators developing new or modifying old antifungal susceptibility tests need to optimize testing conditions to facilitate detection of both antifungal susceptibility and resistance.

In Vitro Susceptibility Testing Factors

The accuracy and standardization of any in vitro susceptibility test is dependent on factors such as incubation time, incubation temperature, buffer, light, inoculum preparation, inoculum size, medium composition, and MIC endpoint definitions (124, 126, 127). The effect of each of these factors on yeast and mold MICs are shown in Tables 8 and 9, respectively (124).

As filamentous fungi are composed of both hyphal and conidial components (Fig. 4), investigators developing mold susceptibility assays have had to chose between three inocula preparations: conidia only; hyphae and conidia; or hyphae only. The conidial inoculum is preferred by most investigators as conidia are easy to enumerate, facilitating simple inoculum standardization. The use of a conidial inoculum should be questioned, however, for two important reasons. First, hyphae, not conidia, are observed during invasive disease. Use of a conidial inoculum is therefore only appropriate if conidial susceptibility to antifungal drug in vitro is predictive of the susceptibility of hyphae to drug in vivo. Second, patient isolates do not always sporulate (i.e. produce conidia)

Table 8. Influence of testing parameters on the MICs of yeasts[a]

Factor	Polyene Testing (AMB)	Azoles Testing (FLC)
Incubation Time	MIC may increase with increased incubation time	MIC may increase with increased incubation time
Incubation Temperature	No uniform effects on MIC	Lower temperatures may result in lower MIC
Buffer, if not present	MIC is increased	MIC is increased
Light	Prolonged light degrades AMB, increasing MIC	No effect on MIC
Inoculum Size	Increased inoculum size increases MIC	Increased inoculum size increases MIC
Medium	Possible antagonism with sterol or sterol-like substances in medium	RPMI best medium for detecting azole resistance
MIC Endpoint Definition	Uncommon growth trailing and -cidal action make MIC definition clear-cut	Common growth trailing due to static action makes MIC definition variable

[a] Adapted from reference 124.

when cultured in vitro. While one may presume that use of a hyphal inoculum would resolve both of these issues, controversy exists as to the best way to obtain a standardized hyphal inoculum. Hyphae can be obtained in three ways: 1) by scraping the surface of a mature fungal colony and removing conidia by filter or gauze; 2) inoculating a broth medium with conidia inoculum, incubating until mature hyphae are produced and centrifuging to obtain a stock hyphal inoculum; and 3) inoculating conidia inoculum directly into each well or tube of a susceptibility test (in the absence of drug) and

Table 9. Influence of testing parameters on the MICs of filamentous fungi

Factor	Polyene Testing (AMB)	Azoles Testing (ITC)
Incubation Time	MIC may increase with increased incubation time	MIC may increase with increased incubation time
Incubation Temperature	No uniform effects on MIC	Lower temperatures may result in lower MIC
Light	Prolonged light degrades AMB, increasing MIC	No effect on MIC
Inoculum Preparation	Hyphae may have higher MIC values than conidia	Hyphae may have higher MICs than conidia
Inoculum Size	Increased inoculum size increases MIC	Increased inoculum size increases MIC
Medium	Am3 medium may allow detection of AMB resistance while RPMI does not	RPMI best medium for detecting azole resistance
MIC Endpoint Definition	Uncommon growth trailing and -cidal action make MIC definition clear-cut	Common growth trailing due to static action makes MIC definition variable

incubating until hyphae develop, at which time drug would be added. Although the first two methods are most commonly used by investigators, it is important to point out that while these hyphal inocula are standardized on the basis of optical density, they are composed of randomly fragmented hyphae of varied lengths. These methods therefore provide no standardization of hyphal condition. The third method of hyphal inoculum preparation seems the most effective way of preparing a hyphal inoculum because every

susceptibility test well is inoculated with the same concentration of conidia, resulting in similar hyphal growth in each well or tube (i.e. standardized hyphal inoculum) and the resulting hyphae remain undisturbed (non-fragmented) during subsequent drug addition. Unfortunately, an important disadvantage of the second and third methods of hyphal inoculum preparation is that they require conidia for well/tube or broth inoculation, respectively, which is impractical for non-sporulating isolates. In vitro susceptibility studies comparing conidial and hyphal MIC values have been limited in number, with varied results. Manavathu (128), Pujol (129), and Espinel-Ingroff (130)

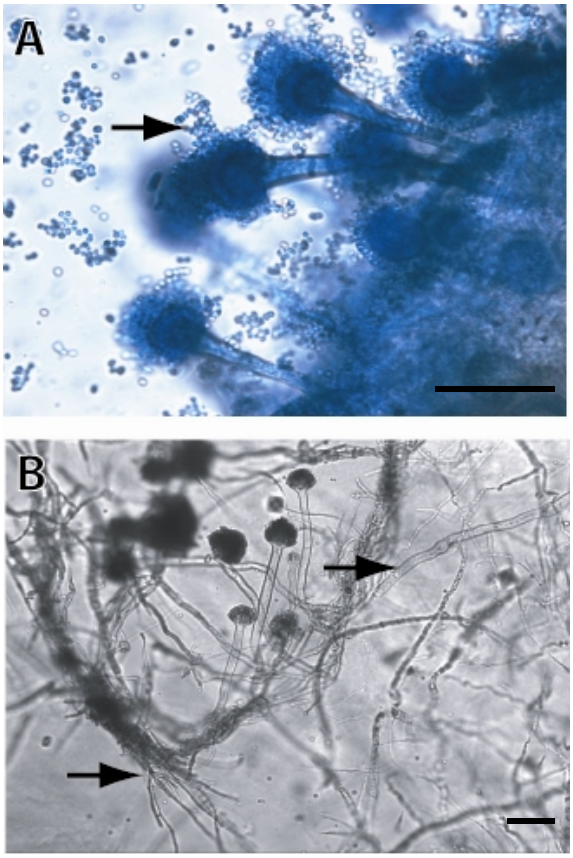


Figure 4. Conidial and hyphal components of *A. fumigatus*. An arrow indicates conidia in (A). Arrows indicate hyphae in (B). Bar represents 100 µm.

reported no significant differences between the conidial and hyphal MIC values while Lass-Florl (131, 132) and Guarro (133) reported significant differences. However, according to both Lass-Florl (134) and Pujol (129), this discrepancy may be due to an inoculum concentration effect, with excessively concentrated or diluted hyphal inocula concentrations resulting in erroneously high or low MIC values, respectively.

Testing factors of any susceptibility testing format can be optimized by using appropriate yeast or mold control strains. These controls must be clinical isolates, originally obtained from patients with confirmed mycoses and a documented history of antifungal therapy and clinical outcome. As both pharmacokinetic effects (poor drug penetration or distribution, unexpected interaction with additional drug, etc.) and underlying disease in the host may be responsible for an unsuccessful clinical outcome, strains obtained from patients with clinical failure (i.e. in vivo resistant) must be reconfirmed as in vivo resistant by testing the strain sensitivity to drug in an appropriate animal mycosis model. Assuming clinical response is directly related to the in vivo susceptibility of a fungus to antifungal therapy, strains isolated from patients with unsuccessful or successful clinical outcomes should have elevated or non-elevated in vitro MIC values, respectively. Optimal susceptibility testing factors will therefore facilitate an MIC reflective of the strain's in vivo susceptibility.

In Vitro-In Vivo Correlation of Current Antifungal ASTs

As stated previously, the ultimate goal of antifungal AST's is the ability to use in vitro MICs as predictors of in vivo clinical outcome. This in vitro – in vivo correlation can only be demonstrated by the ability to establish in vitro MIC breakpoint values. A

breakpoint value is a μg/ml concentration of drug that can be used as a cutoff for MIC interpretation. A MIC greater or less than the breakpoint value predicts clinical resistance or susceptibility, respectively. Breakpoint values can be established by analyzing either the in vitro MICs of isolates obtained from random case study and clinical trial patients with documented therapeutic outcomes or the serum drug concentrations in patients. According to the former method, the final μg/ml breakpoint value will be greater than the μg/ml MICs of isolates obtained from patients with successful clinical outcomes (in vivo-sensitive) and equal to or less than the μg/ml MICs of isolates obtained from patients with unsuccessful clinical outcomes (in vivo-resistant). In the latter method the final μg/ml breakpoint value will be equal to the concentration of drug in serum, with MICs greater than or equal to or less than the breakpoint value indicating resistance or susceptibility, respectively. Unfortunately, caveats of the latter method are that 1) drug concentrations in patient sera continually fluctuate, and 2) drug concentrations are often different in tissue versus serum, with no standardized method of detecting tissue drug levels. Breakpoint values were recently published for FLC, ITC, and 5-FC NCCLS M27-A2 testing of *Candida* spp. (135). However, the NCCLS M38-A assay does not yet provide azole breakpoint values for molds. In addition, no AMB breakpoint values have been unanimously agreed upon for either yeast or molds. The primary reason that AMB breakpoint values have not been defined is that the MICs of in vivo-AMB resistant strains are not consistently greater than MICs of in vivo-AMB sensitive strains (136-139). One could hypothesize that this is due to the pharmacokinetic and/or underlying disease factors present in vivo but not in vitro. However, testing by

Johnson et al. has demonstrated that an in-vivo AMB resistant strain (NCPF 7097), verified as in-vivo AMB resistant using a mouse aspergillosis model, does not produce elevated AMB MICs in vitro (136). This suggests that either NCPF 7097's AMB resistance can only be detected in vivo or that current testing factors and/or methods of in vitro susceptibility testing are not optimized for detection of AMB resistance.

The fact that breakpoint values are only available for FLC, ITC, and 5-FC testing of *Candida* spp. greatly impacts the value of antifungal susceptibility assay in the clinical setting. Without breakpoint values, susceptibility testing should not be performed, as the physician can not justifiably utilize the MIC result in determining the most efficacious antifungal therapy for the patient. Unfortunately, this has not been the case in recent years, with many physicians ordering susceptibility testing and attempting to interpret their results. This is alarming, as misinterpretation of MIC results may result in selection of an antifungal drug for life-threatening infections that does not provide optimal efficacy or toleration. In cases where breakpoint values have not yet been determined in prospective clinical trials, appropriate therapy and antifungal resistance can often be guided by species identification and careful monitoring of the patient's overall condition, respectively.

In Vitro Susceptibility Testing Methodologies

Early attempts in antifungal ASTs were unstandardized, with comparisons between different assays resulting in MIC results varying as much as 50,000-fold (140, 141). By 1983, the National Committee for Clinical Laboratory Standards (NCCLS) recognized the need for standardization and established a subcommittee to develop

standardized susceptibility testing procedures. The NCCLS developed a yeast susceptibility test in 1992, currently designated as the NCCLS M27-A2 (135). This was followed in 1998 by the development of a mold susceptibility assay, currently designated as the NCCLS M38-A (142). Although NCCLS testing introduced standardization, it has major weaknesses, including the subjectivity or observer-bias of MIC determinations and the inability to detect AMB resistance. Despite these weaknesses, and the development of improved ASTs and ARTs, the NCCLS M27-A2 and M38-A assays remain gold-standards for in vitro susceptibility testing of yeast and molds, respectively.

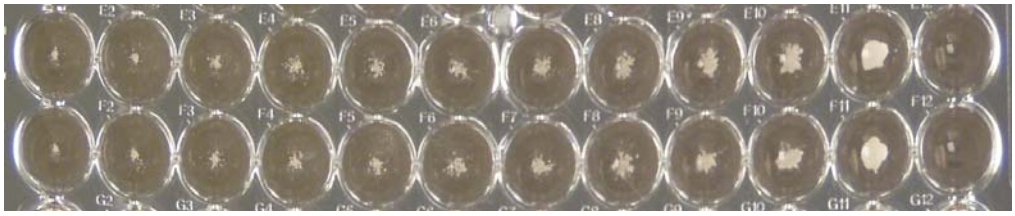
A number of alternative ASTs and ARTs have recently been developed to overcome the shortcomings of NCCLS testing, with improvements in one or more of the following areas: added convenience (reduced initial set-up time, eliminate need for antifungal drug dilutions, etc.; decreased testing time; generation of quantitative (i.e. objective) MICs; and phenotypic detection of azole resistance. These tests employ a number of techniques including NCCLS-based colorimetric assays, agar-diffusion, flow cytometry, and polymerase chain reaction (PCR) based technologies. Only those testing methods relevant to this thesis will be discussed in detail.

NCCLS M27-A and M38-A Methods Both NCCLS M27-A2 and M38-A testing can be performed in either macrodilution or microdilution format. The more accepted microdilution format requires 12 wells in a 96-well microtiter plate to test the susceptibility of an organism to a two-fold dilution series of drug. The highest concentration of drug in well one (64 μg/ml FLC or 16 μg/ml AMB, ITC, VRC, CAS) is

followed by nine two-fold dilutions of the drug. Well eleven contains the growth control (no drug) and well twelve contains medium only as a sterility control.

The NCCLS M27-A2 assay utilizes a 2x yeast inoculum of 1 x 10^3 to 5 x 10^3 colony forming units/ml (CFU/ml), prepared by diluting a 0.09-0.11 optical density (OD_{530nm}) yeast suspension 1:1000. The yeast inoculum is added to wells one through eleven, followed by incubation at 35º C for 48 h. MICs are determined visually at 24 and 48 h. By comparing growth in the growth control well to that in the drug wells, the MIC can be designated as the lowest concentration of drug that results in significant inhibition of organism growth. Significant inhibition is defined differently depending on the drug tested. The AMB MIC endpoint is defined as 100% inhibition of growth due to AMB's fungicidal action and the absence of trailing growth, i.e. partial inhibition of growth over a range of drug concentrations (Figure 5). Due to the fungistatic action of azoles on yeast and observable trailing growth, the azole MIC endpoints are defined as a prominent

A



B

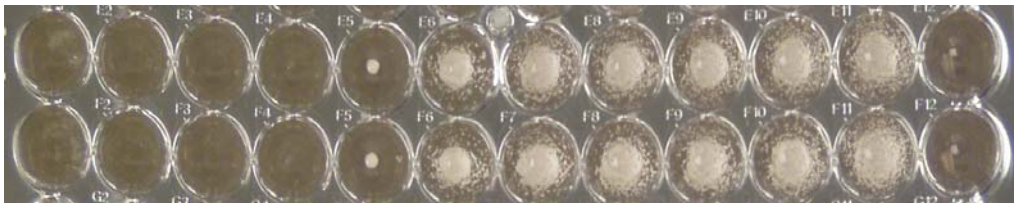


Figure 5. The presence (A) or absence (B) of trailing growth in NCCLS M27-A2 testing. (A) FLC testing with trailing growth in wells 1-7. (B) AMB testing demonstrating no trailing growth.

decrease in turbidity or 50% reduction in growth.

The NCCLS M27-A assay was directly adapted to facilitate development of the NCCLS M38-P assay, for susceptibility testing of filamentous fungi such as *A. fumigatus*, *A. terreus*, *Rhizopus* spp. and *Fusarium* spp. (143). Now in an approved form, the NCCLS M38-A assay differs from the NCCLS M27-A2 in two aspects, inoculum preparation and ITC MIC end-point definition. The NCCLS M38-A inoculum is approximately ten-fold more concentrated than the NCCLS M27-A2 inoculum and is prepared with conidia, not yeast. The ITC MIC endpoint of 100% is higher in M38-A testing due to the fungicidal activity of ITC on molds (144).

Rapid Susceptibility Assay The Rapid Susceptibility Assay (RSA) is an in vitro microdilution assay that was developed to rapidly and objectively determine the susceptibilities of medically significant yeast spp. to AMB and FLC (145). The RSA is based on the hypothesis that when susceptible fungi are exposed to antifungal drug, their consumption of glucose will be suppressed or inhibited. An objective MIC can be generated by measuring levels of residual glucose in the medium containing drug and comparing it to that of controls without drug. As evaluation of glucose utilization is a more sensitive method of MIC determination compared to the NCCLS M27-A2's method of growth observation, it is not surprising that: RSA AMB and FLC MICs can be determined at 19 h, compared to 48 h for the NCCLS M27-A; and RSA AMB and FLC MICs demonstrate high (100%) agreement (± one drug dilution) with NCCLS M27-A MICs.

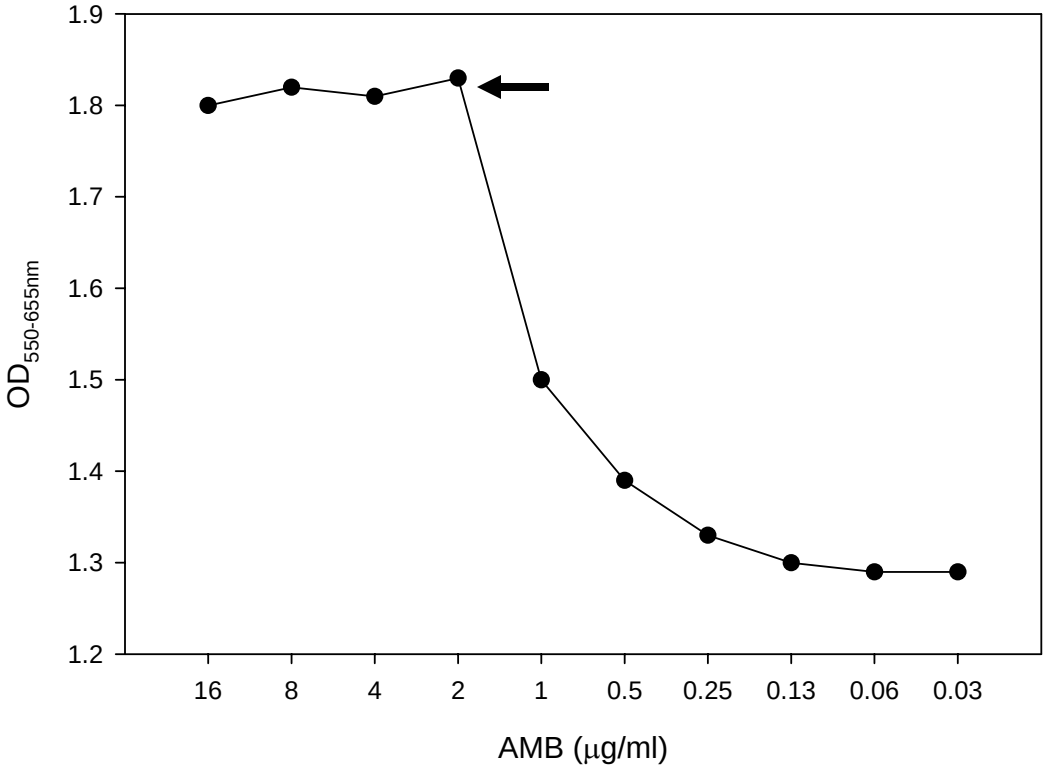


Figure 6. Yeast RSA susceptibility curve and determination of MIC. Antifungal susceptibility curves are generated for each RSA by plotting the percent residual glucose values (y-axis) against the appropriate drug concentrations (x-axis). The MIC is determined by first calculating a detection interval with upper and lower limits represented, respectively, by the $OD_{550\text{-}655nm}$ values corresponding to the glucose control and drug-free growth control. RSA MICs are defined as the lowest drug concentration having an $OD_{550\text{-}655nm}$ value within the top 10% of the detection interval (indicated with an arrow in this example).

RSA testing is performed in duplicate, with a single test consisting of twelve wells in a 96-well microtiter plate (ten testing wells and two controls). Testing wells contains 100 µl of a 3 x 10^6 – 5 x 10^6 CFU/ml yeast-RPMI inoculum, appropriately diluted (dilution dependent on yeast species and antifungal drug tested), and 100 µl of RPMI containing the appropriate 2x dilution of a two-fold drug dilution series. The growth control well is the same as the testing wells but without antifungal drug. The

glucose control well contains neither yeast nor drug. After incubating for 19 h at 35-37º C, residual glucose values are determined colorimetrically by adding 50 µl of a glucose oxidase enzymatic mix (146, 147), incubating at room temperature for 20-30 min, and reading at $OD_{550\text{-}655nm}$ in a spectrophotometer. Objective MICs are obtained by plotting residual glucose values against the corresponding concentration of drug and analyzing the resulting susceptibility curve (Fig. 6).

AMB and FLC RSA conditions were optimized for *C. albicans*, *C. parapsilosis, C. krusei*, and *C. tropicalis* using NCCLS M27-A quality control strains: ATCC 24433 (*C. albicans*); ATCC 90028 (*C. albicans*); ATCC 750 (*C. tropicalis*); ATCC 6258 (*C. krusei*); ATCC 22019 (*C. parapsilosis*); and ATCC 90018 (*C. parapsilosis*). Although these are clinical strains, there is no documentation as to the therapeutic outcomes of the patients from which they were obtained. Until such clinical strains are tested, the ability of RSA MICs to predict in vivo clinical outcome must be questioned. The RSA can also be improved by optimizing conditions for *C. glabrata* testing, introducing testing of new antifungal agents such as VRC and CAS, and modifying conditions to facilitate rapid and objective MIC determination for filamentous fungi.

Elipsometer Test The Elipsometer test (Etest) is an agar-diffusion form of susceptibility testing that is attractive due to its simplicity, reproducibility, and lack of requirement for specialized equipment (143). An Etest plastic strip, impregnated with 15 dilutions of drug, is placed on an RPMI agar plate (2% glucose) previously streaked with a yeast or mold inoculum, with diffusion of drug from the strip into the agar. A zone of growth inhibition, in the shape of an ellipse, can be observed at 24-48 h, with the vertical

length of the ellipse dependent on the organism's susceptibility to drug and the horizontal width dependent on the diffusion properties of the drug in the strip. The MIC is read as the lowest drug concentration at which the border of the elliptical inhibition zone intercepts the scale on the strip. Overall, yeast and mold Etest MIC values have compared favorably (± 1 to 2 drug dilutions) with MIC values obtained with NCCLS M27-A2 and M38-A testing, respectively. The percent agreement between the E-test and NCCLS M38-A MICs of filamentous fungi such as *A. fumigatus*, *A. terreus*, *A. flavus*, *Fusarium* spp., and *Scedosporium* spp., ranges from 20 to 100%, depending on antifungal drug (148-151). The percent agreement between the Etest and NCCLS M27-A2 MICs of yeasts such as *Candida* spp. and *C. neoformans* ranges from 0 to 100%, with again, the percentage dependent on antifungal drug (152-155). For both yeasts and mold, the lowest percent agreement always corresponds to AMB testing. This difference in AMB MIC values between Etest and NCCLS testing may be explained in two ways: first, the use of a higher conidial or yeast inoculum concentration in the E-test method results in erroneously high MICs by 48 h incubation, requiring that AMB MICs be read at 24 h (151, 156); and second, comparisons of Etest and NCCLS M27-A2 AMB MICs of in vivo-AMB resistant clinical isolates of *Candida* spp. and *Cryptococcus neoformans* suggest that the Etest may facilitate detection of AMB resistance while the NCCLS M27-A2 does not (157-160). It is important to point out, however, that the Etest has not yet been demonstrated to detect AMB resistance in filamentous fungi.

Other Colorimetric Methods MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diprenyl-2H-tetrazolium bromide) and XTT (2,3-bis(2-methoxy-4-nitro-5-dulfophenyl)-5-

[(phenylamino) carbonyl]-2H-tetrazolium hydroxide) have been used as colorimetric markers in microdilution susceptibility testing of *Candida* spp. (161-165), *C. neoformans* (162, 163), and mold spp. such as *Aspergillus*, *Fusarium*, and *Scedosporium* (165-168). Both MTT and XTT are reduced by mitochondrial dehydrogenases of metabolically active fungi to a formazan derivative (169). The only difference between the assays is that the MTT assay requires an additional extraction step to solubilize the formazan derivatives. Both assays utilize a NCCLS-based testing format (i.e. identical growth medium, inoculum procedure, incubation conditions, etc.), with XTT and MTT addition occurring immediately after the 24 or 48 h incubation at 35º C. The percent agreement between XTT/MTT and NCCLS testing MICs is excellent (>90%).

The Sensititre Yeast One colorimetric antifungal susceptibility panel utilizes the colorimetric marker Alamar Blue, another oxidation-reduction indicator, which changes color from deep blue to bright pink in the presence of growing organisms or cells (170). The application of Alamar Blue in antifungal susceptibility testing has been demonstrated with *Candida* spp. (165, 171-174), *Cryptococcus* spp. (172, 173), and *Aspergillus* spp. (156, 165, 175). The azole and polyene MICs of *Candida* spp. obtained using Alamar Blue have a high percentage agreement (≥ 90%) with those obtained in NCCLS M27 testing. In contrast to *Candida*, percent agreement between Alamar Blue and NCCLS M38 testing azole and/or polyene MICs of *Aspergillus* spp. are highly variable, often due to differences in incubation time and endpoint definitions between studies.

Optimal Antifungal Susceptibility and/or Resistance Testing

Although antimicrobial susceptibility or resistance testing can be performed in hospital and reference laboratories, most antifungal testing is currently performed in references laboratories. This is unfortunate as the advantage of testing in a hospital laboratory is that it can be initiated on-site as soon as the fungus is identified (ART) and isolated (AST) from a patient sample. The turnaround time for testing results can be much slower when performed at a reference laboratory, as the isolated fungus must first be sent from the hospital laboratory to the reference laboratory. As the chance of a successful clinical outcome inversely correlates with the time it takes to administer an effective antifungal regimen, it is critical to generate MIC data as rapidly as possible. If reference laboratories are to continue as the primary setting for antifungal susceptibility and/or resistance testing, it is important that they begin to use and/or develop testing formats that facilitate MIC determinations in a more rapid manner than would be possible in a hospital laboratory setting. If this is not possible, an appropriate testing format should be introduced to and routinely performed in the hospital laboratory.

Determining the optimal susceptibility and/or resistance testing format for a hospital or reference laboratory requires identification of each method's advantages and disadvantages (Table 10). An ideal testing format in the hospital laboratory would be one that: 1) limits instrument use, as most hospitals can not afford to buy expensive equipment that is used only a small portion of the time; 2) requires limited technical training, as it may be difficult and/or expensive to train all personnel in the hospital laboratory; 3) produces objective MICs, to eliminate the subjectivity of MIC

interpretations between personnel; and 4) requires the least amount of time, to ensure optimal therapy for the patient. Based on Table 10, resistance testing such as PCR and real-time PCR, and susceptibility testing such as flow cytometry are not optimal due to expensive instrumentation and extensive technical training. Although ASTs like NCCLS M27-A2, NCCLS M38-A, and Etest require little instrumentation and technical training, MIC determination can be subjective (especially azole drug testing) and may require ≥ 48 h. The optimal testing format would therefore be one of the colorimetric assays, such as the RSA, XTT, Alamar Blue, which require little instrumentation and provide objective MICs in less than 48 h. Of the three, the RSA is the least time-consuming, generating MICs in the shortest period of time.

As reference laboratories employee a limited number of trained personnel to repeatedly perform the same test daily with large numbers of fungal isolates, an ideal testing format would be one that has the high thorough-put and provides objective MICs. ART testing such as PCR and real-time PCR would be logical in this setting as resistance can be detected in a short period of time, with most PCR and real-time equipment facilitating simultaneous testing of multiple isolates. However, because PCR-based testing may not detect all types of resistance, reference labs should also perform AST testing. Although currently used in reference laboratories, ASTs such as NCCLS M27-A2. NCCLS M38-A, and E-test are still suboptimal due to prolonged incubation times and subjective MIC determinations. Again, a colorimetric assay, such as the RSA, would be the optimal AST format for the reference laboratory.

Table 10. Advantages and disadvantages of in vitro AST and ART formats

Method	Yeast or Mold Testing	Advantages	Disadvantages
NCCLS M27-A2	Yeast	Low Equipment Cost	Subjective MICs Incubation time may exceed 48 h Does not detect AMB resistance
NCCLS M38-A	Mold	Low Equipment Cost	Subjective MICs Incubation time may exceed 48 h Does not detect AMB resistance
RSA	Yeast	Objective MICs Requires less time than NCCLS (19 h)	Requires colorimetric reaction Does not detect AMB resistance
Etest	Yeast	Rapid Set-up May detect AMB resistance	Subjective MICs Requires up to 48 h
	Mold	Rapid Set-up	Subjective MICs Requires up to 48 h
XTT, MTT Alamar Blue	Yeast and Mold	Objective MICs	Require colorimetric reaction Does not detect AMB resistance Requires 48 h
Flow Cytometry	Yeast and Mold	Rapid Results (~6 h)	High equipment cost Requires technical expertise Does not detect AMB resistance
PCR	Yeast and Mold	Rapid Results	Requires specific primers High equipment cost Requires technical expertise Does not detect AMB resistance
Real-time PCR	Yeast and Mold	Very Rapid Results (1-3 h)	Use of specific primers High equipment cost Requires technical expertise Does not detect AMB resistance

Concurrent and Sequential Antifungal Combination

Susceptibility Testing of *Aspergillus fumigatus*

Introduction

With the rapidly increasing incidence, high mortality rates, and antifungal resistance of fungal infections, the development of new concurrent antifungal combination regimens have advantages as they may 1) enhance the rate and extent of fungicidal effect through drug additivity or synergism, 2) increase the spectrum of antifungal activity, 3) facilitate a more rapid antifungal effect; 4) decrease antifungal toxicity, and 5) prevent antifungal resistance (176). While combination therapy has become routine in treating bacterial infections such as tuberculosis, Q fever, and enterococcal bacteremia (177), advances in antifungal combination therapy lag behind, with the only routine combination therapy being the treatment of cryptococcal meningitis with AMB and 5-FC (60, 62, 178).

Sequential antifungal therapy has recently become an important issue due to the advent of new antifungals, regular use of antifungal prophylaxis, and emergence of antifungal resistance. Patients not responding to primary IA therapy, due to host factors or antifungal resistance, can now be treated with secondary or even tertiary antifungal regimens. In addition, patients on antifungal prophylaxis that develop breakthrough IA will also be given secondary and sometimes tertiary antifungal regimens. To confound matters, sequential therapy is often considered combination therapy, as the most common first-line IA therapy, AMB, has a long half-life (≤15 days), resulting in residual levels of primary drug in tissue during subsequent antifungal regimens (50, 53). Therefore, the

greatest concern in sequential therapy is determining the most appropriate and safe sequence of antifungal agents.

Antifungals currently approved to treat the majority of *A. fumigatus* invasive aspergillosis (IA) infections target three cell functions: ergosterol production (VRC, ITC), cell membrane integrity (AMB), and cell wall integrity (CAS). If one also considers the antifungal drugs currently in clinical trials and improved medications of traditional drugs (liposomal AMB and oral and i.v. formulations of ITC), there are a large number of possible concurrent and sequential antifungal combinations that could be used to treat IA. This discussion will be limited to concurrent and sequential antifungal combination therapy and susceptibility testing involving AMB, ITC, and VRC, as the experiments discussed in this thesis were restricted to the use of these three antifungal agents.

Combination therapy may result in pharmacokinetic or pharmacodynamic interactions (176). Pharmacokinetic interactions are those affecting the amount, rate, and ratio of drug concentration at the infection site in vivo. Indirect pharmacokinetic interactions occur when addition of a second drug results in improved penetration or accumulation at the site of infection over a single drug. Direct pharmacokinetic interactions occur when one drug enhances or attenuates the activity of the other by interfering with its adsorption, metabolism, or elimination. Pharmacodynamic interactions can be classified into four groups: 1) those affecting the spectrum of antifungal activity; 2) the rate or extent of killing though synergistic or antagonistic effects; 3) the selection of resistant organisms; and 4) antifungal toxicity. Combining

antifungal drugs to increase the spectrum of antifungal activity may be most suitable in prophylactic antifungal therapy, where a variety of fungi, with varying susceptibilities to any one drug, could potentially cause disease. For example, bone marrow transplant recipients are routinely treated prophylactically with fluconazole to prevent candidiasis (179), yet up to 30% of these patients become infected with *Aspergillus*, a mold refractive to fluconazole therapy (3). The development of a prophylactic regimen of fluconazole combined with a drug effective against *Aspergillus* spp. should perhaps be considered for patients in this high risk group. Combination therapy is usually administered to get a synergistic effect. Synergism is a positive interaction, where the combined effect of the drugs in vivo is significantly greater than the effects of the drugs when used independently. When combination therapy does not result in a significantly greater or less effect than the independent effects of the drugs, the combination effect is termed additive or indifferent. The worst type of combination therapy is one that results in antagonism, a negative interaction in which the combined effect of the drugs in vivo is significantly less than the effects of the drugs when used independently. Combination therapy may also reduce the emergence of resistant strains that cause clinical failure. By administering antifungal drugs in combination, the chance of an invasive *Aspergillus* species becoming resistant to both antifungals is theoretically very low. Combination therapy can also decrease antifungal toxicity by permitting administration of lower doses of toxic drugs like AMB.

Although limited in number, the efficacies of concurrent and sequential antifungal combinations against *Aspergillus fumigatus* have been investigated and/or demonstrated

in three formats: in vitro susceptibility testing with *Aspergillus* clinical or laboratory isolates, in vivo combination therapy in animal IA models; and clinical combination therapy in random IA case studies. Clinical applications of antifungal therapy have been anecdotal, using combinations that have a theoretical advantage (180). While most of these cases involved desperately ill patients who were refractory to standard monotherapy, this practice should be discouraged at this time as preclinical investigations followed by appropriately designed clinical trials are necessary to determine the safety and efficacy of any antifungal combination therapy (180, 181).

In Vitro Antifungal Combination Susceptibility Testing Methods

In vitro antifungal combination susceptibility testing has been developed to rapidly determine the pharmacokinetic interactions between antifungals in vitro (176, 182, 183). It is important to point out that in vitro testing only determines the interactions between drugs at the level of the fungal cell and that interpretation of the results of these interactions do not necessarily correlate with clinical outcome as most cases of antimicrobial synergism in vitro have not been predictive of superior clinical efficacy (184).

Checkerboard Combination Testing

Due to its convenience, checkerboard testing is the most common method used to test antifungal combinations in vitro. A dilution series of one drug is tested against the dilution series of a second drug in a microwell or tube "checkerboard" format, with each well or tube containing a unique combination of antifungal drugs. The concentrations of drug in each dilution series range

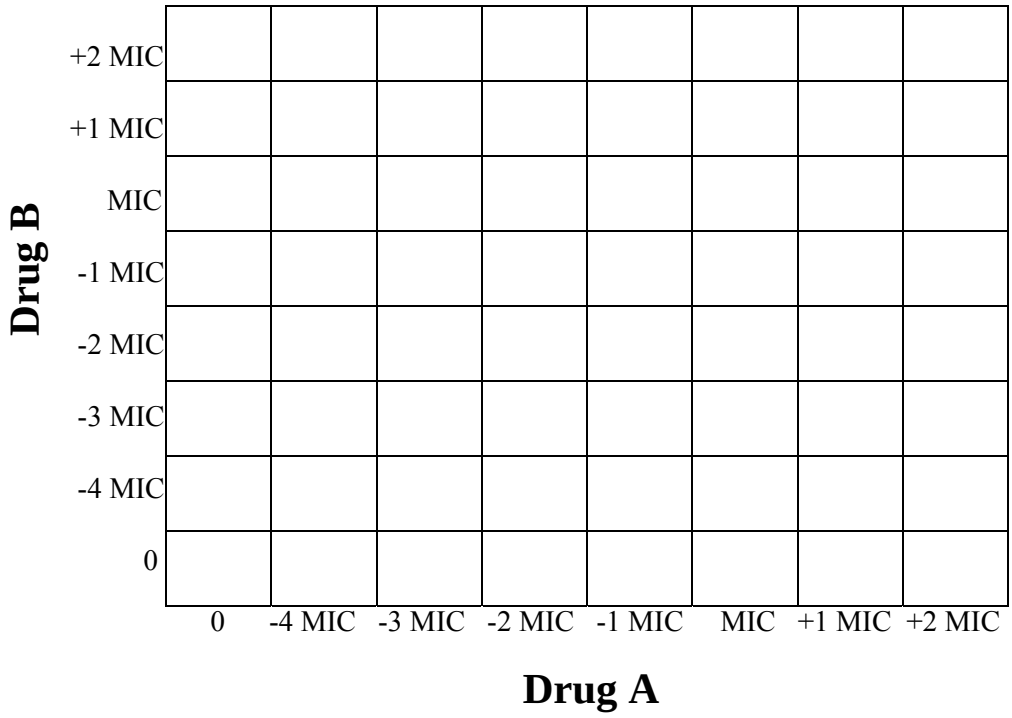


Figure 7. Drug combination set-up in checkerboard testing. A two-fold drug dilution is prepared for each drug that ranges from 4 two-fold concentrations less than to 2 two-fold concentrations greater than the known MIC drug concentration.

from four to five dilutions below the MIC to two dilutions or greater above the MIC (Figure 7). As individual drug MIC data are required for tube/microwell set-up, susceptibility testing with each drug independently must be performed prior to initiating combination testing. The tubes or wells containing drug combinations are inoculated with yeast or mold inocula and incubated for 24 to 48 h at 35-37º C. Before the drug interactions can be analyzed, a MIC endpoint must first be chosen to distinguish wells containing inhibitory versus non-inhibitory combinations of drugs. Most investigators use a growth-based MIC endpoint such as 100% (MIC-0), 80% (MIC -1), or 50%

(MIC-2) growth inhibition depending on the drugs tested. The disadvantage of using one or more of these endpoints is that they introduce the same subjectivity of observer bias already discussed with monodrug susceptibility testing. However, like single-drug susceptibility testing, this bias may be eliminated by performing combination testing in a format that generates objective data, such as Alamar blue, XTT, or RSA testing.

The fractional inhibitory concentration (FIC) index is the most common method used to analyze drug interactions. The FIC of each drug is determined by dividing the concentration of drug necessary to inhibit growth in a given row or column by the MIC of the test organism to that drug alone. The FIC index is calculated by adding the individual FIC values of each drug present in the well or tube (184). Based on the FIC index value, a synergistic, additive, indifferent, or antagonistic interaction can be assigned. Although commonly used, the FIC index has several major disadvantages. When testing a fungistatic and fungicidal drug, there may be a question as to which MIC endpoint should be used. In NCCLS M27-A2 and RSA testing, fungicidal drugs such as AMB have MIC endpoints of 100% growth inhibition while fungistatic drugs such FLC have MIC endpoints of 50-80% growth inhibition. As the use of different endpoints may alter the FIC index interpretation, one solution is to use two FIC indices, one determined with each MIC endpoint (167). The second disadvantage of the FIC index is that checkerboard testing results in multiple FIC values when only one can be chosen as the index value (Fig. 8). Depending on the FIC index chosen, both synergistic and antagonistic interaction effects can often be observed in a single test. Choosing the "correct combination" appears to be a matter of personal choice and is dependent on the

goals of the study. Many investigators use the mean FIC value from the entire range of FIC values as their FIC index (185). Others designate the FIC index as the highest or lowest FIC value from a range of FIC values within clinically relevant concentrations of each drug. A third option is to use the lowest FIC value if none of the other FIC values indicate antagonism or chose the highest FIC value if one or more FIC values indicate antagonism (167). The third disadvantage of the FIC index is that there are numerous definitions described in the literature for its interpretation (Table 11) (167, 185-199).

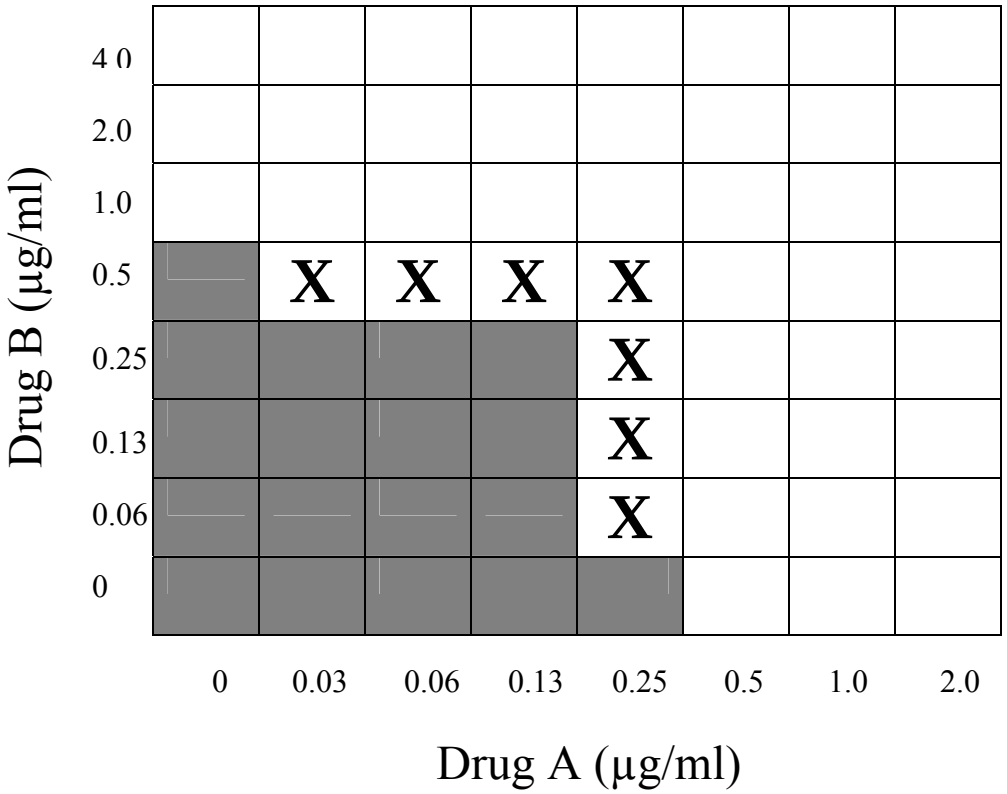


Figure 8. Multiple FIC index values in a single checkerboard test. Grey wells correspond to fungal growth greater than the MIC endpoint definition while white wells correspond to fungal growth equal to or less than the MIC endpoint definition. FIC index values would be determined in wells indicated with an X.

The first set of FIC index definitions shown in Table 10, originally described by Berenbaum in 1978, has been used extensively in assessing both antibacterial and antifungal drug interactions (200). Although widely used, this set of definitions may be too restrictive as it does not recognize indifferent interactions. The second set of FIC index definitions is preferable over Berenbaum's as it includes indifferent interactions and takes into consideration the experimental error of ±1 dilution that is often observed in checkerboard testing (183, 201). The remaining FIC index definitions have been used sparingly in random antifungal combination studies.

Table 11. Published FIC index interpretation definitions

Definition number	FIC Index Interpretation				References
	Synergy	Additivity	Indifference	Antagonism	
1	<1.0	1.0	-	>1.0	146, 112, 191, 140
2	≤0.5	>0.5 - ≤1.0	>1.0 - ≤4.0	>4.0	162, 37, 116, 114, 183, 209
3	<1.0	1.0	>1.0 - ≤2.0	>2.0	187, 210
4	≤0.5	>0.5 - <1.0	≥1.0 - <4.0	≥4.0	141
5	<1.0	≥1.0 - ≤2.0	2.0	>2.0	117
6	≤0.5	> 0.5 – 2.0	-	>2.0	147
7	≤0.5	-	>0.5 - ≤2.0	>4.0	206

Response surface statistical analysis is an alternative method for analyzing drug interactions (202). Originally utilized in antiviral combination studies (203, 204), the response surface approach considers the results in every well, fits a model to the whole response surface, and provides a quantitative interaction value with 95% confidence intervals. The E-max based, fully parametric Greco model is a response surface model that fits the entire data set with a nonlinear regression analysis and calculates an objective interaction coefficient alpha (ICα) that represents the drug interaction. If the ICα is zero, greater than zero, or less than zero, the drug interaction is interpreted as additive, synergistic, or antagonistic, respectively. Statistical significance of the ICα is determined by analyzing its 95% confidence interval (CI). If the CI includes zero, the drug interaction is not significant and should be considered additive. If the interval does not include zero, the drug interaction is considered significant and the original ICα interpretation is upheld. The one limitation of the Greco model, at least to investigators using a NCCLS-based endpoint, is that the model requires quantitative input data for each well. Observer-based endpoints (NCCLS) are therefore not permitted due to their subjectivity. This limitation has been addressed by the use of colorimetric assays such as MTT to objectively quantify the level of growth (167).

Time-Kill Combination Testing Time-kill combination testing is another method used to test antifungal combinations in vitro. Unlike the checkerboard method, which can only show fungistatic activity, time-kill testing measures the fungicidal activity of the combination being tested. This makes the time-kill testing format more relevant for clinical situations in which fungicidal therapy is desired. Another advantage is that time-

kill testing provides a dynamic picture of antifungal interaction and interaction over time (based in CFU/ml), as opposed to the checkerboard, which is examined at one time point. Unfortunately, the constant CFU/ml determinations are tedious and greatly limit the number of antifungal concentrations and combinations that can be tested (184).

Concurrent Antifungal Combination Therapy and In Vitro Susceptibility Testing

Amphotericin B and Itraconazole The most controversial concurrent antifungal combination is an azole with AMB (azole/AMB). The greatest debate has been whether the azole inhibits ergosterol production, thereby eliminating AMB's target and resulting in antagonism. While this would be a major concern if the azole acted on the fungal cell prior to AMB, it is in reality the opposite, with AMB acting on the fungal cell before the azole (176). Other hypotheses for azole/AMB antagonism involve the rapid accumulation of lipophilic azoles on the fungal cell surface, inhibiting AMB binding to ergosterol (205) or that the early effects of AMB on the cell membrane interferes with azole entry into the cell (206).

In an excellent review of 249 cases of concurrent combination therapy, Denning et al. reported 41 cases of patients treated with AMB and ITC (AMB+ITC). While the 21 deaths may have occurred due to antagonism, the remaining patients all demonstrated clinical improvement. Results of AMB+ITC therapy in mouse and guinea pig IA models have resulted in predominantly indifferent effects. The results of in vitro AMB+ITC susceptibility testing are just as confusing, with 1, 4, and 2 studies indicating synergy, additivity, and antagonistic effects, respectively (207). Clearly, the current results of both

in vivo and in vitro AMB+ITC studies are too inconsistent to make a statement regarding the true effects of AMB+ITC.

Amphotericin B and Voriconazole Little has been documented regarding the in vivo and in vitro effects of combining AMB and VRC (AMB+VRC). We could not locate any literature reporting AMB/VRC clinical use or the results of in vivo AMB/VRC animal studies. Manavathu et al. demonstrated an indifferent AMB/VRC effect in vitro by a checkerboard technique that measured ^{14}C-amino acid incorporation by mycelia for determination of fungal growth (208).

Itraconazole and Voriconazole We were unable to locate any literature in which the in vivo or in vitro effects of concurrent ITC and VRC were presented. This was expected as ITC and VRC are proposed to have the same fungal target.

Sequential Antifungal Combination Therapy and In Vitro Susceptibility Testing

Amphotericin followed by Itraconazole or Voriconazole The most documented sequential therapy is AMB followed by ITC (A→I). This is not surprising as AMB has, until recently, been the primary therapy for patients with IA. As ITC was the only other antifungal regimen available, it was administered as the secondary therapy if AMB therapy failed or was terminated due to toxicity issues. In fact, a widely accepted regimen uses AMB until neutropenia resolves followed by ITC maintenance therapy (207, 209). This sequence of therapy has been repeatedly documented as causing no harm and in some cases being more efficacious than either AMB or ITC monotherapy (209-

212). As VRC is a relatively new drug, information regarding its use after AMB (A→V) has been limited to a few case studies. However, these examples demonstrate patient improvement when VRC is initiated following discontinuation of AMB (213-215).

Reports of A→I and A→V in vitro susceptibility testing are very limited. Using checkerboard testing, Maesaki et al. showed that a 1 h pretreatment of *A. fumigatus* conidia with AMB, followed by 24 h incubation with ITC, resulted in greater synergistic effects than those obtained when the drugs were administered concurrently (216). Maesaki et al. later performed additional A→I in vitro susceptibility testing, this time using mycelial weight as an indicator of inhibition, and found no synergistic effect by pretreating with AMB (217). Importantly, no antagonistic effects were noted in either study.

Itraconazole or Voriconazole followed by Amphotericin B The most controversial sequence of antifungal therapies is an azole followed by AMB. Although some case studies have described patients improving with primary and secondary regimens of ITC and AMB (ITC→AMB), most patients receiving ITC→AMB therapy either die or are given a tertiary therapeutic regimen (218-225). There have been no clinical reports of failed VRC therapy followed by AMB therapy.

Both in vivo and in vitro studies have investigated the effect of ITC→AMB. Lewis et al. used a mouse model of sinopulmonary aspergillosis to determine the effect of ITC→AMB therapy in vivo. Antagonism was demonstrated using four different endpoints: CFU/ml in lung tissue; whole-lung chitin assay; mortality at 96 h; and histopathology of lung sections (226). Using both in vitro checkerboard and mycelial

weight testing formats, Maesaki et al. found that pretreatment with ITC prior to AMB resulted in antagonism (216, 217). Schaffner and Bohler also demonstrated ITC→AMB by performing time-kill studies (227). The consensus of both in vivo and in vitro studies is that ITC→AMB results in antagonism.

Two hypotheses have been proposed to explain azole→AMB antagonism. Although neither has been tested with *A. fumigatus*, preliminary testing has been conducted with *C. albicans*. The most popular hypothesis is that the azole inhibits ergosterol production, resulting in the loss of AMB's target. Vazquez et al. tested this hypothesis by adding ergosterol to the *C. albicans* growth medium during incubation in the presence of FLC. If FLC was responsible for preventing ergosterol synthesis, replacement of the lanosterol derivatives with ergosterol should have negated antagonism following addition of AMB. Unfortunately, *C. albicans* grown in FLC-medium containing the highest levels of ergosterol were as resistant to AMB as *C. albicans* grown in FLC-medium containing no ergosterol. Either ergosterol was not being incorporated into the cell membrane or inhibition of ergosterol synthesis is not the only reason for azole→AMB antagonism (207, 228). The second hypothesis is that lipophilic azoles like ITC block the interaction between AMB and ergosterol by absorbing to the cell surface. Scheven et al. found that azole-induced depletion of ergosterol in the cell membrane of *C. albicans* required at least 1 h, but that azole bound ergosterol could be replaced by approximately 6 h (205).

Itraconazole followed by Voriconazole Very little has been documented about the in vivo effects of ITC followed by VRC (ITC→VRC), and no information is

available regarding the in vivo or in vitro effects of VRC followed by ITC. Case studies describing ITC→VRC therapy have demonstrated patient improvement (229, 230).

Dissertation Hypotheses

The first four dissertation hypotheses pertain to monodrug antifungal susceptibility testing of various yeasts and molds, while the last three hypotheses pertain to concurrent and sequential antifungal combination susceptibility testing of *A. fumigatus*.

Hypothesis 1

The development of the yeast RSA was a landmark in antifungal susceptibility testing as it provided objective AMB and FLC MICs in 19 h, while the current gold-standard antifungal susceptibility method, the NCCLS M27-A, provided subjective MICs and required 48 h. I hypothesize that the yeast RSA can be modified to determine antifungal susceptibilities of filamentous fungi, and predict that the mold RSA (mRSA) will provide a more rapid and/or objective alternative to Etest and NCCLS M38-A testing.

Hypothesis 2

Because hyphae are the morphological form of *A. fumigatus* observed in vivo during invasive aspergillosis infections, it is necessary to determine whether the use of hyphal inocula is more appropriate than conidial inocula in in vitro antifungal susceptibility testing to obtain clinically significant MICs. I hypothesize that the use of a hyphal inoculum in mRSA testing (hRSA) will result in significantly different antifungal

MICs than those obtained with the standard conidial inoculum. If correct, this will imply that all antifungal susceptibility testing be performed using hyphal inocula.

Hypothesis 3

The ability of the yeast RSA to generate clinically significant FLC MICs for *C. albicans* has not been demonstrated. I hypothesize that this can be demonstrated by performing RSA FLC testing with clinical *C. albicans* clinical strains with known clinical outcomes.

Hypothesis 4

Due to the increasing incidence of *C. glabrata*-related candidiasis, it is essential that the yeast RSA have the capability to perform susceptibility testing with this species. I hypothesize that yeast RSA conditions can be optimized for *C. glabrata* testing using clinical *C. glabrata* strains with known clinical outcomes.

Hypothesis 5

There is increasing interest in concurrent and sequential antifungal therapy as a means to obtain synergistic drug effects, decrease drug toxicity, and delay emergence of antifungal resistance. Unfortunately, published data on in vivo and in vitro antifungal interactions are sparse. I hypothesize that the mRSA can be modified to facilitate concurrent and sequential antifungal (AMB, ITC, VRC) combination testing of conidial inocula of *A. fumigatus* clinical isolates. These results will be essential in deciding which antifungal combinations should be tested in an invasive aspergillosis animal model.

Hypothesis 6

This hypothesis is essentially the same as hypothesis 5, except that I will be determining the susceptibilities of *A. fumigatus* hyphae to concurrent and sequential antifungal (AMB, ITC, VRC) combinations by modifying the hRSA. I hypothesize that the in vitro effects of antifungal combinations on hyphae will be significantly different than the in vitro antifungal combination effects on conidia. If true, this will imply that hyphal inocula should be used instead of conidial inocula in combinatorial antifungal susceptibility testing.

Hypothesis 7

The use of FIC indices in interpreting antifungal combination effects has a number of disadvantages that can be eliminated by interpreting combinatorial effects via response surface statistical analysis. As the use of this method requires quantitative data, I hypothesize that the quantitative residual data generated by mRSA- and hRSA-modified antifungal combination testing can be used as imput data in the response surface computer program, ModLab, to determine the effects of antifungal combinations on *A. fumigatus* conidia and hyphae, respectively.

CHAPTER 2

MODIFICATION OF THE YEAST RAPID SUSCEPTIBILITY ASSAY FOR ANTIFUNGAL SUSCEPTIBILITY TESTING OF *ASPERGILLUS FUMIGATUS*

Introduction

The rapid susceptibility assay (RSA) was previously developed by Riesselman et al. for antifungal susceptibility testing of yeasts such as *Candida albicans* (145). The assay is based on the assumption that when fungi are exposed to an inhibitory concentration of antifungal drug, their uptake of nutrients, such as glucose, is suppressed. A minimum inhibitory concentration (MIC_{RSA}) value is determined by comparing the residual glucose levels in the medium following incubation of the fungus with and without drug. This approach provides objective, quantitative MIC values. The MIC_{RSA} values for yeast compare favorably with the National Committee for Clinical Laboratory Standards (NCCLS) M27-A antifungal susceptibility assay, but the latter uses a subjective endpoint of growth inhibition and requires more time (231).

Several compelling reasons favor adapting the RSA for use in mold susceptibility testing. First, the incidence of morbidity and mortality due to infections caused by opportunistic filamentous fungi, such as *Aspergillus fumigatus*, is increasing (232). Second, emergence of resistance during antifungal therapy has been documented (206, 233). Third, the introduction of voriconazole (90), caspofungin acetate (84), and liposomal formulations of amphotericin B has increased therapy options. Fourth, current

susceptibility testing for filamentous fungi, such as the NCCLS M38-P assay, suffers from the same limitations as those cited for the yeast M27-A assay (234).

To address these issues, we have modified the RSA to facilitate susceptibility testing for *A. fumigatus*. Conidia were chosen as the inoculum type, in accordance with other susceptibility tests, with additional modifications of initial glucose concentration, inoculum concentration and MIC determination. Glucose utilization was monitored during conidial germination and hyphal development in the presence or absence of amphotericin B (AMB) and itraconazole (ITC), with AMB and ITC MIC_{RSA} value determinations based on the percent residual glucose. To determine whether the RSA has advantages over the NCCLS M38-P assay in terms of time and objectivity, AMB and ITC MIC_{RSA} values obtained at 16, 24, and 48 h were compared to MIC_{NCCLS} values determined at 48 h according to the NCCLS M38-P guidelines. We show that by using a 0.11 OD_{530nm} inoculum, *A. fumigatus* AMB and ITC MIC_{RSA} values obtained at 16 h were equal to or within a single two-fold dilution of AMB and ITC MIC_{NCCLS} values obtained at 48 h. Preliminary testing with *A. terreus* also showed that by using a 0.11 OD_{530nm} inoculum, AMB and ITC MIC_{RSA} values obtained at 16 h were equal to or within a single two-fold dilution of AMB and ITC MIC_{NCCLS} values obtained at 48 h.

Materials and Methods

Reagents

Preparations of RPMI 1640 without glucose (Sigma, St. Louis, MO, cat. no. R-1383) or with 0.50 to 16 mg/ml glucose were buffered with 0.165 M 3-(N-morpholino)-

propanesulfonic acid (MOPS) (USB, Cleveland, OH, cat. no. 19256) and adjusted to pH 7.0 with 10 N NaOH.

Fungal Strains

A. fumigatus strains NCPF 7102, 7101, 7100, 7098, and 7097 were purchased from the National Collection of Pathogenic Fungi (Mycology Reference Laboratory, Bristol). These strains were isolated from aspergillosis patients with known clinical outcomes. *A. terreus* 55 and *A. fumigatus* 46 were clinical strains donated by the University of Virginia Health System. Species designations of *A. fumigatus* and *A. terreus* strains were confirmed by observations of macroscopic and microscopic characteristics during growth on Czapek-Dox agar (35). All stocks were maintained in vials of sterile water at 4º C.

Antifungal Agents and Dilutions

Amphotericin B (AMB) (Sigma A-9528) and itraconazole (ITC) (Research Diagnostics Inc., Flanders, NJ, cat. no. R51211) were purchased in powdered form. Stocks of 3200 µg/ml were prepared in dimethyl sulfoxide (DMSO) (Sigma D-2650) and stored at -20º C for up to 6 months. For both RSA and NCCLS M38-P testing, serial twofold dilutions were prepared following NCCLS M38-P guidelines (234), except that the final 2x concentration range of 0.06 to 32 µg/ml AMB and ITC was prepared in 0.4% glucose RPMI 1640. Both drug-free growth and glucose controls were also prepared, containing the same ratio of DMSO to 0.4% RPMI 1640 as the 2x drug dilutions.

Microwell Plate Preparation

Each NCCLS M38-P and RSA test was performed in duplicate in 96 well round bottom plates (Costar, Corning, NY, cat. no. 3799) with each row, comprised of 12 wells, corresponding to one susceptibility test. All testing was performed at least three times on separate days. Wells 1-10 were inoculated with either 100 μl of the 2x AMB or ITC dilution range (0.06 to 32 μg/ml). Wells 11 and 12, the drug-free growth control and glucose control, respectively, were inoculated with 100 μl of 0.4% glucose-DMSO RPMI 1640. Plates were prepared on the day of use and stored at 4º C until needed.

Inoculum Preparation

Isolates were cultured on Sabouraud glucose agar (SAB) (BBL, Cockeysville, MD, cat. no. 211584) slants at 30º C for 3 days (*A. fumigatus*) or 7 days (*A. terreus*). After flooding with glucose deficient RPMI 1640, the slant surface was gently scraped with a sterile wooden applicator stick. After the heavier hyphal fragments had settled, approximately 15 min, the conidial suspension was collected and adjusted with glucose deficient RPMI 1640 to an optical density (OD_{530nm}) of 0.46, 0.26, or 0.11. The 0.11 OD_{530nm} density conidial preparation is referred to as the RSA density conidial inoculum (CI_{RSA}).

NCCLS M38-P recommended conidial inocula (234) used in NCCLS M38-P and RSA testing were prepared by diluting the CI_{RSA} 1:50 with glucose-deficient RPMI. The 1:50 diluted conidial preparation is referred to as the NCCLS density conidial inoculum

(CI_{NCCLS}). All conidial inocula were kept on ice after preparation and used within three hours.

RSA

The RSA was initiated by inoculating wells 1-11 with 100 µl of CI_{RSA} or CI_{NCCLS}, well 12 with 100 µl glucose deficient RPMI and incubating at 35-37º C. Residual glucose levels were detected by addition of 50 µl of an enzyme color mix comprised of 0.6 M sodium phosphate buffer (pH 6.0), 360 µg/ml of 4-amino antipyrine (4-AAP) (Sigma A4382) , 490 µg/ml of N-ethyl-N-sulfopropyl-m-toluidine (TOPS) (Sigma E8506), 0.68 U/ml horseradish peroxidase (Sigma P8415), and 0.4 U/ml glucose oxidase (ICN Biochemicals, Aurora, OH, cat. no. 195196) to each well. At 20 min, the color intensity was measured at OD_{550nm} with a reference reading of OD_{655nm} in a plate reader (Benchmark Plus, Biorad, Hercules, CA).

RSA Antifungal Susceptibility Curves and MIC Endpoint Determination

The percent residual glucose of each test well was calculated by comparing the OD_{550nm} value of the test well with the OD_{550nm} of the glucose control well based on the following equation: (OD_{550nm} of test well / OD_{550nm} of glucose control well) X 100. The percent inhibition of glucose utilization due to AMB or ITC was determined by the following equation: 1- [(100% - % residual glucose in well containing antifungal drug) / (100% - % residual glucose of growth control)] X 100. Antifungal susceptibility curves were generated for each RSA by plotting the percent residual glucose values (y-axis) against the appropriate AMB or ITC concentrations (x-axis). The resulting detection

interval contained an upper and lower limit represented, respectively, by the highest concentration of antifungal drug and the drug-free growth control. AMB MIC_{RSA} endpoint determinations were defined as the lowest drug concentration having a percent residual glucose value within the top 10% of the detection interval. The ITC MIC_{RSA} endpoint was defined as the lowest drug concentration corresponding to a residual glucose value within the top 20% of the detection interval only if: a detection interval comprised of an upper plateau, steep decline, and lower plateau was ; and the highest concentration of ITC (16 µg/ml) had a corresponding residual glucose value of ≥ 80%. If these conditions were not met, the ITC MIC_{RSA} was designated as >16 µg/ml.

NCCLS M38-P Testing and MIC Endpoint Determination

The NCCLS M38-P assay was initiated by inoculating wells 1-11 with 100 µl of CI_{NCCLS}, well 12 with 100 µl of glucose deficient RPMI and incubation at 35-37º C. Although the AMB and ITC dilutions were prepared in 0.4% glucose RPMI and the conidial inocula were prepared in glucose deficient RPMI, the final concentration of glucose was 0.2% at the time of incubation in accordance with NCCLS M38-P procedures. NCCLS MIC (MIC_{NCCLS}) values were determined by visual inspection at 48 h using a mirror plate reader (Cooke Engineering Co., Alexandria, VA) according to NCCLS M38-P guidelines {470) with AMB MIC_{NCCLS} endpoint values designated as the lowest AMB concentration preventing growth and ITC MIC_{NCCLS} endpoint values designated as the lowest ITC concentration reducing growth by 50% compared to the growth control.

Statistics

Analysis of variance was used for comparison of residual glucose values determined after conidial inocula incubations with 16 μg/ml AMB or ITC for 24 h. A *P* value <0.05 was considered significant.

Results

Optimal Glucose Utilization Conditions

The incubation temperature, initial glucose concentration, and plate shaking conditions facilitating optimal glucose utilization were determined by monitoring the glucose utilization of 0.11, 0.26, and 0.46 0.11 OD_{530nm} conidial inocula of *A. fumigatus* strain 46 during a 12 h incubation. Figures 9, 10, and 11 show the effect of incubation temperature on the glucose utilization of each conidial inoculum concentration. Incubation at room temperature did not result in detectable glucose utilization during the 12 h, while incubation at 30º C and 35º C resulted in detectable glucose utilization after 3 h. The greatest glucose utilization was observed during the 12 h, 35º C incubation, regardless of conidial inoculum concentration.

The glucose concentration facilitating the greatest percentage of glucose consumption by a conidial inoculum in the shortest period of time was considered optimal. A 0.26 OD_{530nm} conidial inoculum of *A. fumigatus* strain 46 was incubated for 8 h at 35º C in the presence of 8, 4, 2, 1, 0.5, or 0.25 mg/ml glucose. The greatest percentage of glucose utilization was observed in the presence of 1 mg/ml

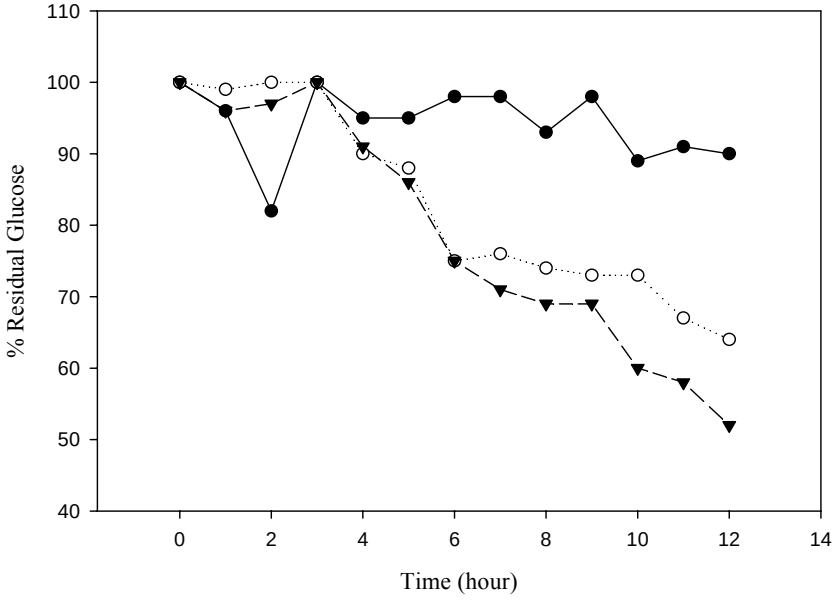


Figure 9. Glucose consumption of 0.11 OD_{530nm} conidial inoculum of *A. fumigatus* strain 46 during 12 h

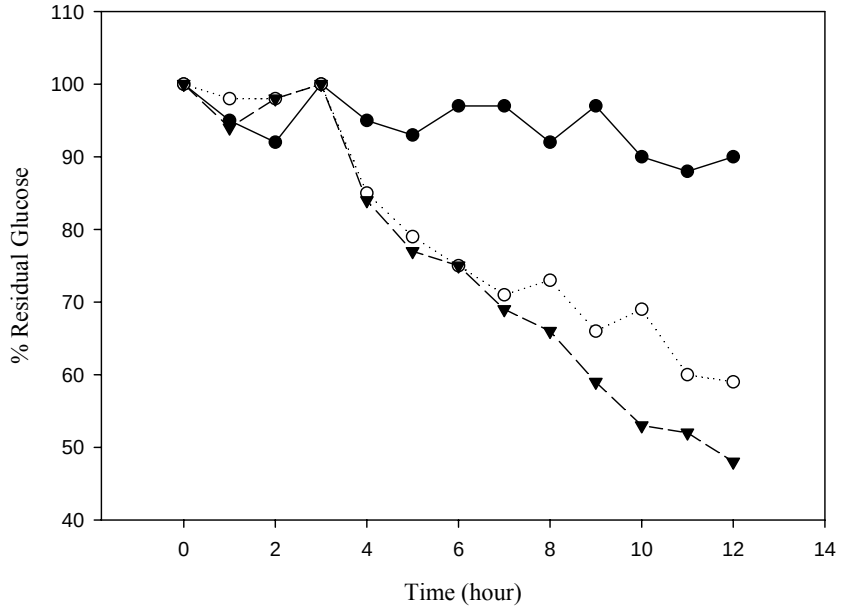


Figure 10. Glucose consumption of 0.26 OD_{530nm} of *A. fumigatus* 46, conidial inoculum during 12 h incubation at room temperature (●), 30º C (○), or 35º C (▼).

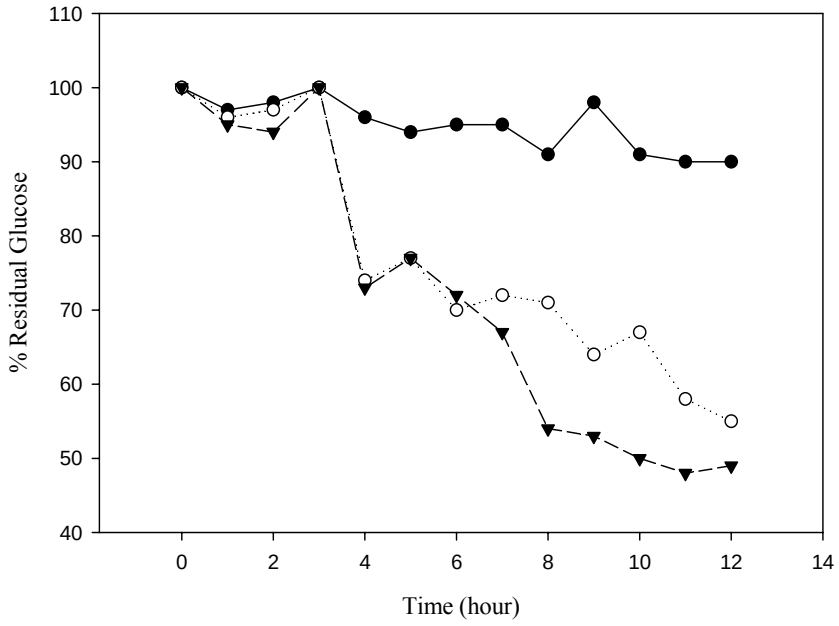


Figure 11. Glucose consumption of 0.46 OD_{530nm} of *A. fumigatus* 46, conidial inoculum during 12 h incubation at room temperature (●), 30º C (○), or 35º C (▼).

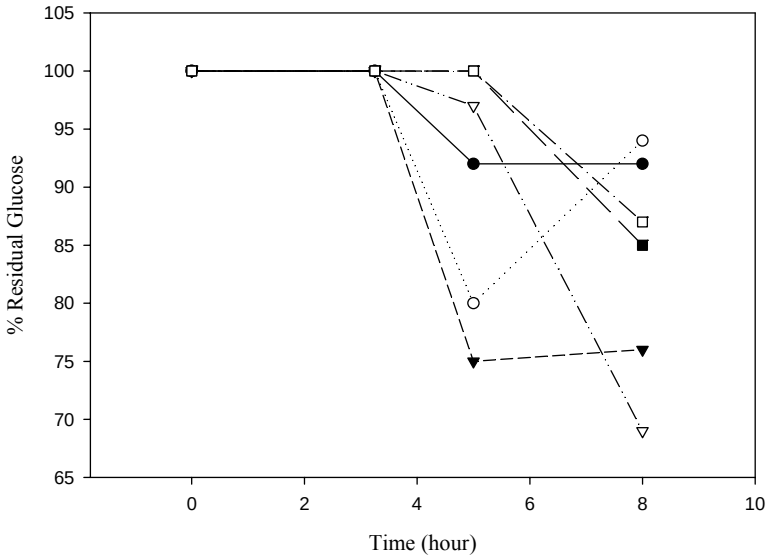


Figure 12. Glucose utilization of 0.26 OD_{530nm} conidial inocula of *A. fumigatus* 46 during 8 h incubation at 35º C in the presence of 8 (●), 4 (○), 2 (▼), 1 (▽), 0.5 (■), or 0.25 mg/ml (□) glucose.

glucose, followed closely by 2 mg/ml glucose (Figure 12).

The influence of shaking on conidial glucose consumption was determined by comparing the glucose utilization of 0.11 or 0.26 OD_{530nm} conidial inocula of *A. fumigatus* strain 46 in the presence and absence of shaking (150 rpm), during a 24 h, 35º C incubation (Figure 13 and 14). Shaking significantly increased the glucose

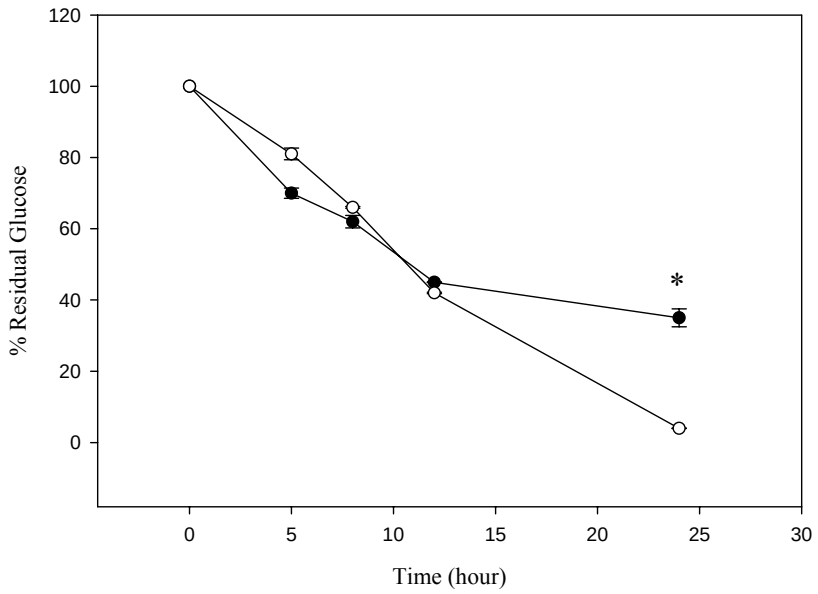


Figure 13. Glucose utilization of 0.11 OD_{530nm} conidial inoculum of *A. fumigatus* strain 46 in the presence (●) or absence (○) of shaking at 150 rpm. Asterisk indicates a significant difference ($P<0.05$) between % residual glucose values in the presence and absence of shaking at that time period.

consumption of both 0.11 and 0.26 OD_{530nm} conidial inocula between 12 and 24 h ($P < 0.05$).

Conidial Growth and Glucose Utilization

To determine the relationship of conidial development to glucose utilization in the absence of antifungal agents, CI_{RSA} were prepared from *A. fumigatus* NCPF strains 7102,

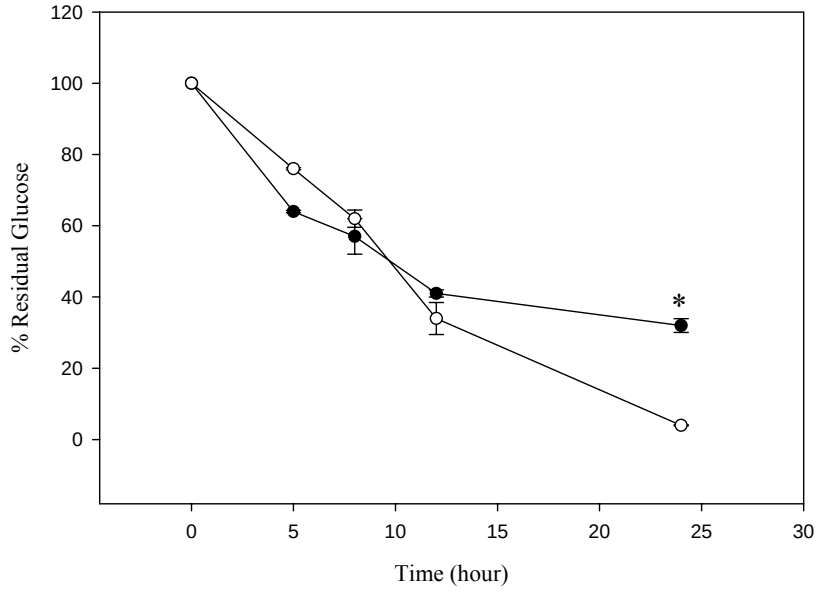


Figure 14. Glucose utilization of 0.26 OD_{530nm} conidial inoculum of *A. fumigatus* strain 46 in the presence (●) or absence (○) of shaking at 150 rpm. Asterisk indicates a significant difference ($P<0.05$) between % residual glucose values in the presence and absence of shaking at that time period.

7101, 7100, 7098, and 7097 and incubated in 0.2% glucose RPMI at 37º C for 24 h. Observations of wet mounts prepared at 0, 3, 6, 8 and 24 h showed that conidia of all strains initiated swelling, germination, and hyphal extension at similar time points (data not shown). Conidial morphology remained unaltered (Fig. 15A) for the first few hours, with conidial swelling becoming clearly evident at 6 h (Fig. 15B). By 8 h, germ tubes emerged (Fig. 15C) and progressed to increasingly more complex hyphal development throughout the remainder of the 24 h incubation (Fig. 15D). The pattern glucose utilization was similar between strains during the 24 h incubation (Fig. 16). Rates of glucose utilization were greatest between 0 and 16 h, with residual glucose levels

decreasing from 100% to 40-50% at 16 h. Decreased rates of glucose utilization were observed between 16 and 24 h with final residual glucose values of 30-40%.

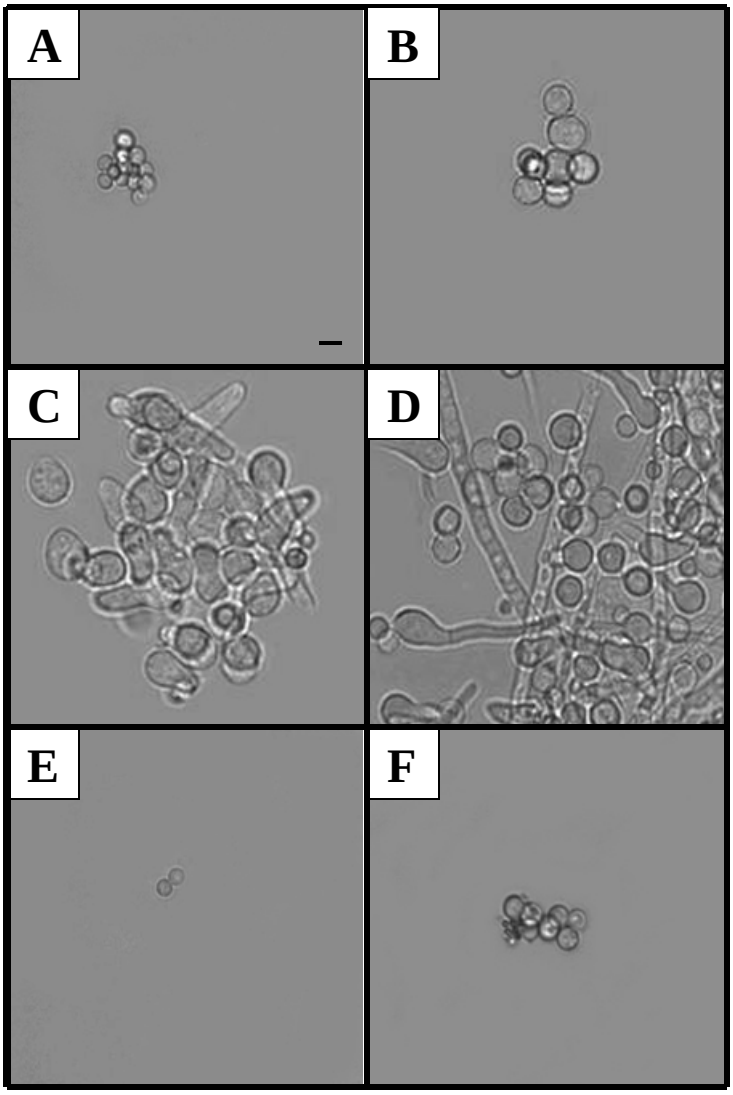


Figure 15. Conidial development during 24 h incubation at 37º C. Wet mount slide preps were observed (400x) at A) 3 h, B) 6 h, C) 8 h, D) and 24 h with no antifungal agent; E) 24 h with 16 µg/ml AMB; and F) 24 h with 16 µg/ml ITC. Bar represents 5 µm.

Antifungal Effect on Conidial Development and Glucose Utilization

To determine the effect of AMB and ITC on conidial development and glucose utilization, CI_{RSA} were prepared with all *A. fumigatus* strains and incubated in 0.2% glucose RPMI containing 16 µg/ml AMB or ITC at 37º C for 24 h. AMB had a similar effect on all strains, with inhibition of conidial development occurring prior to swelling (Fig. 15E) and residual glucose values of 94.3% at 24 h, corresponding to 90% inhibition

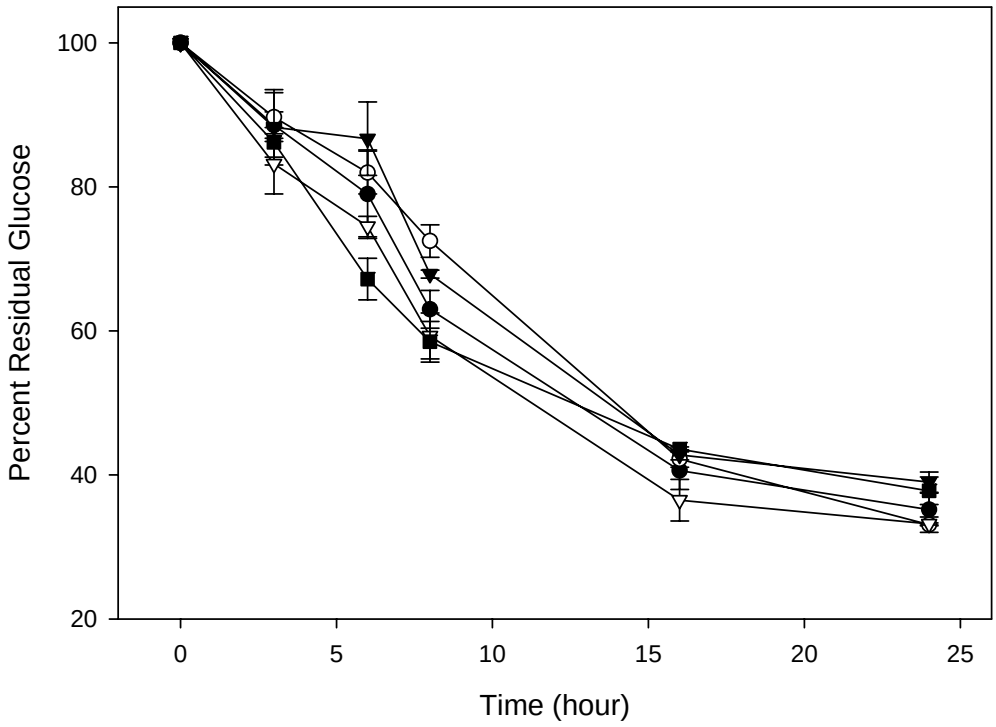
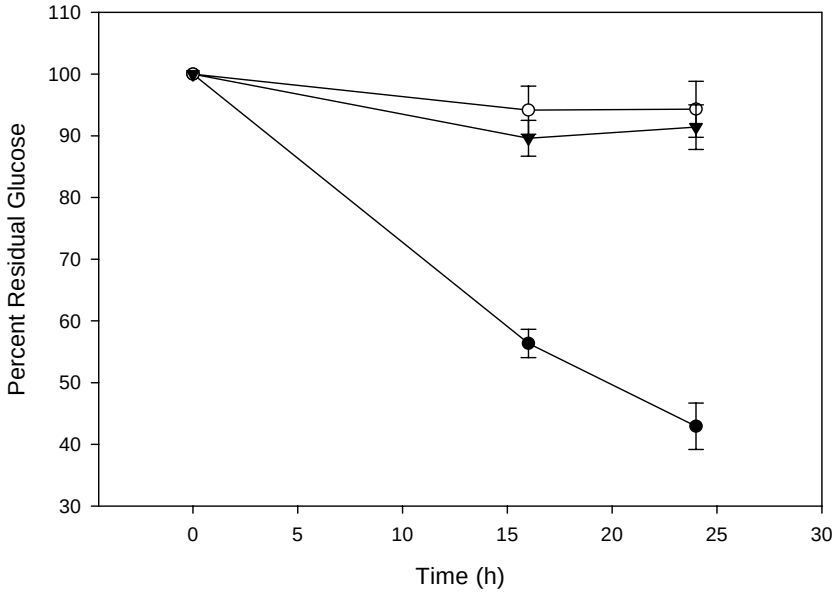


Figure 16. Glucose utilization of *A. fumigatus* strains during 24 h incubation at 37º C. NCPF strains included: 7102 (○); 7101 (●); 7100 (■); 7098(▽); and 7097 (▼).

A Strains NCPF 7102, 7101, 7098, 7097



B Strain NCPF 7100

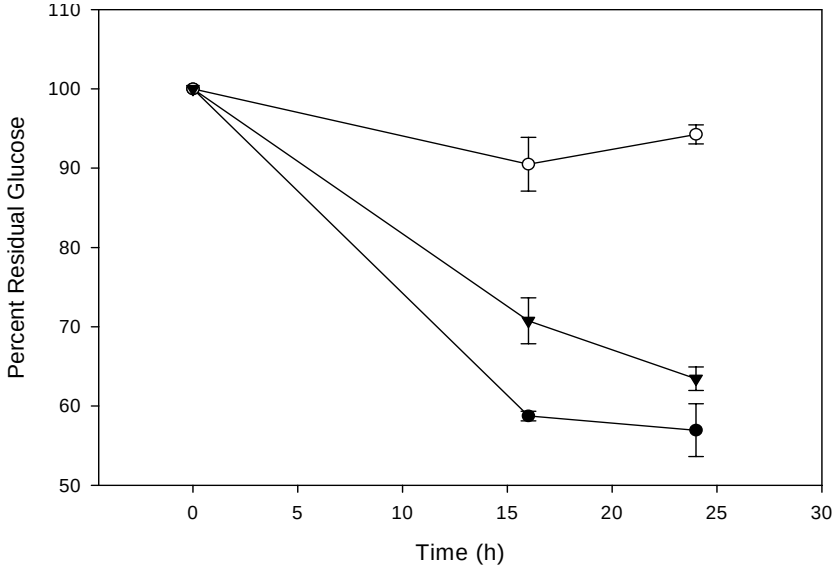


Figure 17. Glucose utilization of *A. fumigatus* strains during 24 h incubation at 37º C in the presence of no antifungal agent, 16 µg/ml AMB, or 16 µg/ml ITC. Average glucose utilization of strains NCPF 7102, 7101, 7098, 7097 (A) in the presence of no antifungal drug (●), 16 µg/ml AmB (○) or 16 µg/ml ITC (▼). Average glucose utilization of strain NCPF 7100 (B) in the presence of no antifungal drug (●), 16 µg/ml AmB (○) or 16 µg/ml ITC (▼).

of glucose utilization (Fig. 17). With the exception of strain NCPF 7100, ITC inhibited conidial development at a partially swollen state (Fig. 15F) with residual glucose values of 91.4% at 24 h, corresponding to 85% inhibition of glucose utilization (Fig. 17A). The ITC concentration of 16 μg/ml was non-inhibitory for strain NCPF 7100, as shown by hyphal development (data not shown) and residual glucose value of 65%, i.e. 22% glucose inhibition, at 24 h (Fig 17B).

Optimization of RSA Conditions

To determine the optimal RSA conditions of incubation time and conidial inoculum, AMB and ITC RSAs were performed with all strains at 16, 24, and 48 h using CI_{RSA} and CI_{NCCLS}. Optimal incubation time and conidial inoculum density conditions were defined as those providing a MIC_{RSA} value equal to or within a single two-fold dilution of a MIC_{NCCLS} value within the shortest period of incubation. Preliminary testing with conidial inocula of 0.26 and 0.44 OD_{530nm} densities provided unsatisfactory results (data not shown) and were not used in optimization experiments. The AMB RSA susceptibility curves for the CI_{RSA} and CI_{NCCLS} of strains NCPF 7101 and 7100 at 16 h were characterized by a detection interval composed of an upper plateau representing inhibitory concentrations of AMB, a steep decline representing the dose-dependent inhibitory range, and a lower plateau representing non-inhibitory concentrations of AMB (Fig. 18). While residual glucose values in the upper plateau remained fairly constant with increased incubation periods, residual glucose values representing the lower plateau decreased with increased incubation periods.

The AMB MIC_{RSA} endpoint was defined as the lowest AMB concentration corresponding to a residual glucose value in the upper 10% of the detection interval. For all strains tested, AMB MIC_{RSA} values obtained at 16 h were equal to or within a single two-fold dilution of MIC_{RSA} values obtained at 48 h regardless of conidial inoculum density (Table 12). More importantly, AMB MIC_{RSA} values obtained with conidial inocula of either density at 16 h were equal to or within a single two-fold dilution of MIC_{NCCLS} values obtained at 48 h.

With the exception of strain NCPF 7100, ITC RSA susceptibility curves (Fig. 19 A, B) of all strains were similar to AMB susceptibility curves, with a detection interval comprised of a drug sensitive upper plateau, dose-dependent sensitivity decline, and drug insensitive lower plateau. ITC MIC_{RSA} endpoints were defined as the lowest ITC concentration corresponding to a residual glucose value in the upper 20% of the detection interval. As with AMB, ITC MIC_{RSA} values at 16 h were equal to or within a single two-fold dilution of MIC_{RSA} and MIC_{NCCLS} values obtained at 48 h (Table 12).

Strain NCPF 7100 showed differences between susceptibility curves for the two inocula densities tested. The CI_{RSA} susceptibility curves had little or no detection intervals, with residual glucose values ≤72% corresponding to 16 μg/ml ITC at 16, 24 and 48 h (Fig 19C). The 16 h CI_{NCCLS} susceptibility curve had a well defined detection interval, with a residual glucose value of 91% corresponding to 16 μg/ml. However, at 24 and 48 hours, detection intervals were indiscernible, with residual glucose values of 66% and 60% corresponding to 16 μg/ml ITC, respectively (Fig. 19D). The CI_{RSA} ITC MIC_{RSA} value of >16 μg/ml at 16, 24, and 48 h was equal to the ITC MIC_{NCCLS} value

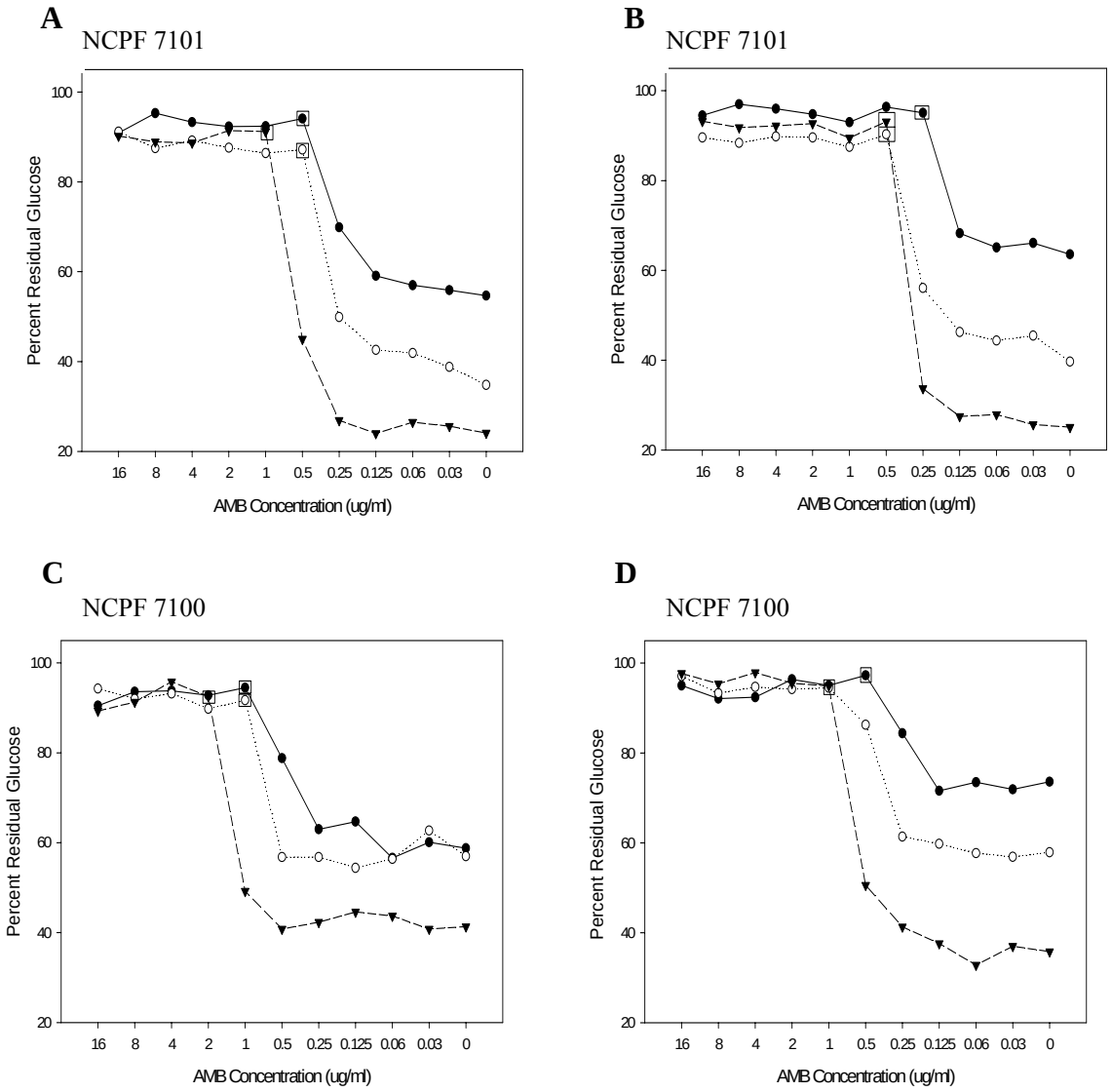


Figure 18. RSA AMB susceptibility curves for strains 7101 and 7100 at 16 (●), 24 (○), and 48 (▼) h in the presence of 0.03-16 µg/ml AMB. CI_{RSA} results are shown in A and C, while CI_{NCCLS} results are shown in B and D. MIC values in each curve are designated with a box. These data represent one experiment. This experiment was performed three times, resulting in similar curves with MIC points equal to or within a single two-fold dilution of the MIC points shown. *A. fumigatus* strains NCPF 7102, 7098, and 7097 gave similar results to NCPF 7101 and 7100.

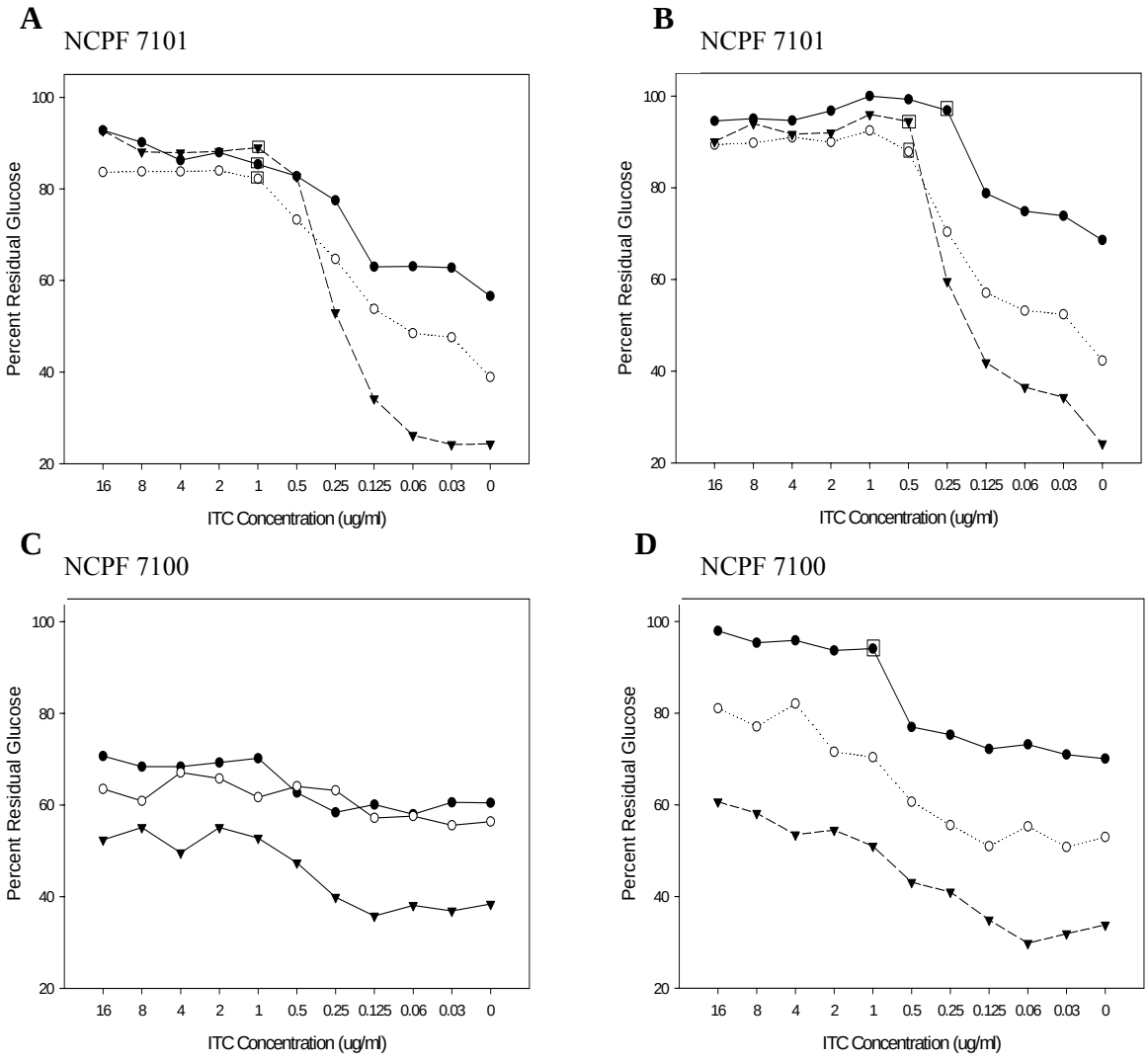


Figure 19. RSA ITC susceptibility curves for strains 7101 and 7100 at 16 (●), 24 (○), and 48 (▼) h in the presence of 0.03-16 µg/ml ITC. CI_{RSA} results are shown in A and C, while CI_{NCCLS} results are shown in B and D. MIC values in each curve are designated with a box if value is ≤ 16 µg/ml. These data represent one experiment. This experiment was performed three times, resulting in similar curves with MIC points equal to or within a single two-fold dilution of the MIC points shown (except CI_{NCCLS} of 7100 at 24 h). *A. fumigatus* strains NCPF 7102, 7098, and 7097 had similar results to NCPF 7101.

determined at 48 h (Table 12). The CI_{NCCLS} ITC MIC_{RSA} value was 1 µg/ml at 16 h but increased to >16 µg/ml by 24 h.

Table 12. RSA (CI_{RSA} and CI_{NCCLS}) and NCCLS M38-P MIC values at 16, 24, and 48 h[a].

Aspergillus spp.	NCPF number	Incubation period (h)	AMB MIC (µg/ml)			ITC MIC (µg/ml)		
			RSA		NCCLS	RSA		NCCLS
			CI_{RSA}	CI_{NCCLS}	CI_{NCCLS}	CI_{RSA}	CI_{NCCLS}	CI_{NCCLS}
A. fumigatus	7100	16	1	0.5		>16	1	
		24	1	1		>16	>16[b]	
		48	2	1	1	>16	>16	>16
	7098	16	0.5	0.25		0.5	0.5	
		24	0.5	0.5		1	0.5	
		48	1	0.5	0.5	1	0.5	0.5
	7097	16	0.5	0.5		0.5	0.5	
		24	0.5	0.5		0.5	0.5	
		48	1	0.5	0.5	0.5	0.25	0.25
	7102	16	0.5	0.5		0.5	0.25	
		24	0.5	0.5		0.25	0.5	
		48	1	0.5	0.5	0.5	0.5	0.25
	7101	16	0.5	0.25		1	0.25	
		24	0.5	0.5		1	0.5	
		48	1	0.5	0.5	1	0.5	0.5
A. terreus		16	0.5	≤0.03		0.25	≤0.03	
		24	1	0.5		0.25	0.125	
		48	4	1	1	0.25	0.25	0.25

[a] These data represent a single experiment. The experiment was repeated two additional times with MIC values equal to or within a single two-fold dilution of results shown.
[b] variable MIC results (1-16 µg/ml)

A. terreus RSA Testing

Preliminary AMB and ITC RSA testing was performed with CI_{RSA} and CI_{NCCLS} of *A. terreus* strain 55 at 16, 24, and 48 h. Use of a CI_{RSA} provided AMB and ITC MIC_{RSA} values at 16 h equal to or within a single two-fold dilution of AMB and ITC MIC_{NCCLS}

values obtained at 48 h while a CI_{NCCLS} required 24 h for AMB and ITC MIC_{RSA} determination (Table 1).

Discussion

The principle objective of this study was to modify the yeast RSA to facilitate antifungal susceptibility testing of *A. fumigatus*. The first step was to determine optimal glucose utilization conditions for 0.11, 0.26, and 0.46 OD_{530nm} conidial inocula. The greatest glucose utilization for all conidial inocula concentrations occurred at 35º C (Fig. 9-11). Although the greatest optimal glucose utilization was obtained with a RPMI glucose concentration was 0.1%, we chose to use the second most optimal glucose concentration of 0.2%, as this was the concentration used in NCCLS M38-P testing (Fig. 12). While plate shaking did significantly increase glucose consumption (Fig. 13, 14), we later found that it increased the time necessary to obtain accurate MIC values (data not shown) and was therefore not used. The relationship of conidial development with glucose utilization was next investigated. Conidia of all strains utilized glucose in a similar pattern, with greatest utilization occurring during periods of conidial swelling and germination (0-16 h) and less utilization once hyphae had developed (16-24 h). When conidia were incubated in the presence of 16 µg/ml AMB or ITC, AMB caused a rapid inhibition as conidia did not develop past the pre-swollen state. However, in the presence of ITC, conidia appeared to develop normally for about 6 h before being inhibited in a partially swollen state. Because AMB has a direct effect on ergosterol, whereas ITC inhibits the ergosterol synthetic pathway (207, 208), the differential inhibitory effect of

the two drugs was not unexpected. This difference was also consistent with inhibition of conidial glucose utilization in the presence of 16 μg/ml of either drug. AMB inhibited 90% of the glucose utilization (94.3% residual glucose) over a 24 h incubation period while ITC inhibited 85% of the glucose utilization (91.4% residual glucose). As these residual glucose values were marginally, but not significantly different (P >0.5), we concluded that ITC inhibited conidial development only a short period after AMB. Since these data suggested that inhibition of growth, the criterion used in NCCLS M38-P testing, could be correlated with inhibition of glucose utilization, we concluded that glucose utilization could be used as an indicator of conidial susceptibility to AMB and ITC.

Yeast RSA testing parameters requiring modification for the mold RSA were inoculum concentration, incubation time, and MIC endpoint determination. Optimization of these conditions was determined by analyzing AMB and ITC susceptibility curves generated from RSA experiments performed at 16, 24, and 48 h using conidial inocula of varying density. Optimal conditions produced susceptibility curves composed of a clearly defined upper plateau (inhibitory doses of drug), a steep decline (dose-dependent inhibition), and a lower plateau (non-inhibitory doses), with few or no residual values located in the dose-dependent decline. Preliminary mold RSA experiments with conidial inocula of 0.11, 0.26, and 0.42 OD_{530nm} densities showed that as conidial inocula density increased from 0.11 to 0.26 and 0.42 OD_{530nm}, the appearance of the susceptibility curves became less defined, with a dose-dependent decline of decreased slope containing increasing numbers of values (data not shown). These data showed that if the conidial

inoculum exceeds a certain density, the glucose discriminatory interval of the mold RSA is compromised. Since preparation of the CI_{NCCLS} for the NCCLS M38-P assay is done by making a 1:50 dilution of a 0.9 to 0.11 OD_{530nm} conidial inoculum (CI_{RSA}) we compared RSA results obtained by using a CI_{RSA} and CI_{NCCLS}. Analysis of AMB susceptibility curves (Fig. 18) for both inocula densities led to several generalizations regarding AMB detection intervals: 1) all susceptibility curves are well defined at each time interval regardless of inoculum density; 2) all conidial inocula of each density produce defined susceptibility curves that are similar at each incubation period; 3) longer incubation periods result in broader detection intervals; and 4) inhibitory concentrations of AMB (located in the upper plateau) at 48 h correspond with residual glucose values in the top 10% of the detection interval at 16 h, regardless of inoculum density. Based on this last observation, the AMB MIC_{RSA} endpoint was defined as the lowest AMB concentration having a percent residual glucose value in the upper 10% of the detection interval. According to this criterion, either inoculum density can be used to obtain AMB MIC_{RSA} values at 16 h.

Analysis of ITC susceptibility curves (Fig 19) for all strains except NCPF 7100 resulted in similar conclusions as AMB susceptibility curves except that inhibitory concentrations of ITC at 48 h corresponded with percent residual glucose values in the top 20% (>83% residual glucose), rather than the top 10%, of the detection interval at 16 h, thus defining the ITC MIC_{RSA} endpoint as the lowest ITC concentration corresponding to a percent residual glucose value in the upper 20% of the detection interval. This criterion could only be used for MIC determination if two other requirements were also

met: 1) the presence of a clearly discernible susceptibility curve composed of an upper plateau, drug-sensitive decline, and lower plateau; with 2) residual glucose values ≥ 80% in the upper plateau. If these conditions were not met, the MIC was designated as >16 µg/ml. These additional requirements were established based on comparisons of the 16 h CI_{RSA} susceptibility curves of strains NCPF 7100 and 7101 (Fig. 19 A, C). The NCPF 7100 susceptibility curve was essentially flat as all ITC residual glucose values were nearly the same as the drug-free control, with a 72% residual glucose value corresponding to 16 µg/ml ITC (Fig. 19C) while strain NCPF 7101 had a defined susceptibility curve with residual glucose values > 80% in the upper plateau (Fig. 19A). As strain NCPF 7100 lacked both a discernable susceptibility curve, with residual glucose values no greater than 80%, its MIC_{RSA} was designated as >16 µg/ml. Comparison of 16 h CI_{RSA} and CI_{NCCLS} susceptibility curves also showed that a CI_{RSA} is superior to the CI_{NCCLS}, as the latter produced an erroneous ITC MIC_{RSA} value of 1 µg/ml at 16 h.

Denning, et al. used strains NCPF 7102, 7101, 7100, 7098, and 7097 repeatedly in agar- and broth-based susceptibility testing studies (136, 237). Strain NCPF 7100 was obtained from a patient who did not respond to ITC therapy and thus the strain was deemed *in vivo* resistant to ITC, whereas NCPF 7098 was obtained from a patient who responded to ITC and the strain was deemed *in vivo* susceptible. Of potential importance is that the MIC_{NCCLS} values obtained by these investigators showed ITC *in vitro* resistance of NCPF 7100 and *in vitro* susceptibility of NCPF 7098, suggesting that *in vitro* MIC testing may have predictive value of therapeutic outcome for aspergillosis

patients treated with ITC. Using similar reasoning to that used for ITC, Denning used the results of susceptibility testing with strains NCPF 7101 and 7097, isolates from aspergillosis patients with failed therapy, to conclude that *in vivo* resistance to AMB does not correlate with *in vitro* MIC_{NCCLS} results. ITC and AMB MIC_{RSA} values obtained in our studies have led to similar conclusions. The CI_{RSA} ITC MIC_{RSA} value of >16 μg/ml for strain NCPF 7100 at 16 h correlates with the clinical history of *in vivo* ITC resistance. We also showed that *in vivo* resistance to AMB did not correlate with *in vitro* resistance as CI_{RSA} preparations of strains NCPF 7102 and 7097 had AMB MIC_{RSA} values of 0.5 and 1 μg/ml at 16 h, respectively, indicating *in vitro* drug susceptibility.

Results of studies with strains NCPF 7102 and 7097 in experimental animals suggest why this discrepancy between *in vivo* resistance and *in vitro* susceptibility may exist (136, 237). Strain NCPF 7102, originally designated as *in vivo* resistant, showed *in vivo* susceptibility in experimental animals, suggesting that host factors may be important in predicting therapeutic outcome. However, strain NCPF 7097 showed *in vivo* resistance in experimental animals, suggesting that current susceptibility testing, including the mold RSA, is thus far unable to detect AMB resistance. Clearly, further work needs to be done to obtain AMB MIC values that are predictive of patient outcome.

Preliminary testing was performed with *A. terreus* to assess whether the RSA could be used with medically significant species other than *A. fumigatus*. As with *A. fumigatus*, only a CI_{RSA} provided AMB and ITC MIC_{RSA} values at 16 h equal to or within a single two-fold dilution of AMB and ITC MIC_{NCCLS} values. While the ITC MIC_{RSA} remained unchanged between 16 and 24 h incubation, it was interesting that the AMB

MIC_{RSA} increased from 0.5 μg/ml at 16 h to 4 μg/ml at 48 h. As we did not observe eight-fold MIC_{RSA} value increases with *A. fumigatus* isolates between 16 and 48 h, we hypothesize that this phenomenon may be due to the slower growth rate of *A. terreus* as compared to *A. fumigatus*. The four-fold difference in the CI_{RSA} MIC_{RSA} and MIC_{NCCLS} values, 4 and 1μg/ml respectively, at 48 h was also an unexpected result. One possibility for this is that the CI_{RSA}**,** fifty-times greater than the CI_{NCCLS}, produced a skewed AMB MIC_{RSA} value. This, however, seems unlikely as use of a CI_{RSA} resulted in ITC MIC_{RSA} values at 16, 24, and 48 h equal to the MIC_{NCCLS} value. Another possibility is that the RSA provides more accurate MIC values than the NCCLS M-38P assay. Numerous studies have suggested that *A. terreus* is naturally resistant to AMB therapy *in vivo* (115, 238)**,** which is more consistent with the 4 μg/ml AMB MIC_{RSA} value than the AMB MIC_{NCCLS} value of 1 μg/ml. The testing of additional *A. terreus* strains with detailed clinical histories and/or animal experimentation data may well help resolve this issue.

In conclusion, these data show that the detection of glucose utilization within a 0.2% glucose RPMI solution can be used to predict the *in vitro* susceptibility of *Aspergillus fumigatus* to AMB and ITC at 16 h incubation at 37º C using an CI_{RSA}. Preliminary RSA testing with the slower-growing *A. terreus* also suggests that ITC and AMB MIC_{RSA} values within a single two-fold dilution of ITC and AMB MIC_{NCCLS} values can be determined by 16 h. This assay is an improvement over the current susceptibility assay as MIC_{RSA} values are quantitative and objective, while the MIC_{NCCLS} values are subjective and rely on observation of growth. Furthermore, the RSA is relatively inexpensive and reduces technician workload as compared to NCCLS M38-P testing.

CHAPTER 3

EXPANSION AND VALIDATION OF MOLD RAPID SUSCEPTIBILITY ASSAY

Introduction

I previously demonstrated that the mold RSA (mRSA) provided objective amphotericin B (AMB) and itraconazole (ITC) minimum inhibitory concentration (MIC) values for *Aspergillus fumigatus* isolates in 16 h, a significant improvement compared to the subjective AMB and ITC MICs obtained by National Committee for Clinical Laboratory Standards (NCCLS) M38-A testing in 48 h. In this current form, however, the mRSA is restricted to AMB and ITC susceptibility testing of *A. fumigatus*. As our ultimate goal is to develop the mRSA for wide scale use as a generalized antifungal susceptibility test for all filamentous fungi, it is essential that I continue to validate current mRSA conditions by testing additional *A. fumigatus* clinical strains, introduce new antifungal drugs to the mRSA drug arsenal, and investigate the suitability of mRSA testing for non-*A. fumigatus* mold species.

mRSA Testing of Non-*A. fumigatus* Mold Species

Aspergillus sydowii, A*spergillus terreus*, *Aspergillus flavus*, *Fusarium oxysporum*, and *Scedosporium angiospermum* clinical isolates were chosen to determine the suitability of mRSA testing for non-*A. fumigatus* molds. These species were chosen based on their availability from Kevin Hazen at the Health Sciences Center in

Charlottesville, VA. The first and most essential step in determining suitability of mRSA testing is to demonstrate a direct correlation between antifungal drug effect on both conidial development and glucose consumption. Establishment of this correlation is dependent on the fulfillment of two conditions: 1) in the presence of an inhibitory concentration of drug, both conidial development and glucose consumption are inhibited; and 2) in the absence of an inhibitory concentration of drug, there is no inhibition of conidial development or glucose consumption. Those species demonstrating a successful correlation will be considered suitable for inclusion in mRSA testing.

Introduction of VRC to mRSA Testing

Voriconazole (VRC) was recently approved by the US Food and Drug Administration for primary treatment of invasive aspergillosis (43, 176). VRC is available in both oral and intravenous forms, with better adsorption and fewer associated side effects compared to AMB and ITC. Like ITC, this triazole targets the cytochrome P450 14α-demethylase enzyme which is involved in ergosterol production (239). Although both ITC and VRC have the same fungal target, cross-resistance has not been consistently observed with ITC-resistant *A. fumigatus* strains, most likely due to target binding differences (109, 241). VRC has demonstrated excellent in vitro activity against *A. fumigatus*, *A. terreus*, *A. flavus*, and *S. angiospermum*, with VRC MICs usually less than AMB MICs and similar to ITC MICs (213). While the in vitro VRC activity against *F. oxysporum* isolates is variable, with MICs ranging from 0.25 to >8 μg/ml (213, 214), VRC prescribed on a compassionate basis (salvage therapy) to patients with AMB and/or

ITC refractory invasive fungal infections caused by *Aspergillus*, *Scedosporium*, and Fusarium spp. has resulted in excellent response rates (215-217). The low toxicity, high adsorption, and broad spectrum of activity, both in vitro and in vivo, warrants inclusion of VRC in mRSA testing.

Validation of mRSA

mRSA ITC and AMB testing conditions were originally optimized with ITC-sensitive and –resistant *A. fumigatus* strains NCPF 7098 and 7100, respectively and AMB-sensitive and resistant *A. fumigatus* strains NCPF 7101 and 7097, respectively (Chapter 2). While testing of the former strains has demonstrated that the mRSA can generate clinically relevant ITC MICs in 16 h, testing of additional ITC-sensitive and ITC-resistant clinical isolates is necessary to reinforce that these conditions are optimal. mRSA and NCCLS M38-A AMB, ITC, VRC testing was performed with four new ITC-resistant and two new AMB-sensitive *A. fumigatus* strains to validate mRSA ITC testing conditions and to confirm that 16 h mRSA MICs are consistently equal to or within one dilution of those obtained by NCCLS M38-A testing in 48 h.

Optimization of *A. flavus* AMB, ITC, and VRC mRSA Testing Conditions

In the first section of results in this chapter, I establish a direct correlation between the effects of AMB and ITC on *A. flavus* conidial development and glucose utilization (Fig. 20-25). Based on this correlation, I will attempt to optimize mRSA conditions for AMB and ITC testing of *A. flavus* strains AFL1, AFL2, and AFL3. As

VRC has also been introduced to the mRSA for *A. fumigatus* testing, I will also attempt to optimize mRSA conditions for VRC testing of the *A. flavus* strains.

Materials and Methods

Reagents

Preparations of RPMI 1640 with or without 0.4% glucose were buffered with 0.165 M 3-(N-morpholino)-propanesulfonic acid (MOPS) and adjusted to pH 7.0 with 10 N NaOH.

Fungal Strains

A. flavus strains AFL1, AFL2, and AFL3; *A. sydowii* strains AS1 and AS2; *A. terreus* strain AT1; *F. oxysporum* strain FO1; and *S. prolificans* strain SC1 were donated by Kevin Hazen at the University of Virginia Health System (Charlottesville, VA). ITC-resistant *A. fumigatus* strains BR181, AF90 (NCPF 7099), AF1422, AF72, and BR130 were donated by David Denning at Hope Hospital, England. AMB-sensitive *A. fumigatus* strains CM1396 and CM237 were donated by Emilia Mellado at the Instituto de Salud Carlos III (Madrid, Spain). ITC-sensitive and –resistant *A. fumigatus* strains NCPF 7098 and 7100, respectively, and AMB-sensitive and –resistant strains 7101 and 7097, respectively, were purchased from the National Collection of Pathogenic Fungi (Bristol, England). *A. fumigatus* strain 204305 was purchased from the American Type Culture Collection (ATCC). Species designation of each strain was confirmed by observations of macroscopic and microscopic characteristics during growth on Potato-

Dextrose and/or Sabouraud (SAB) agar. All stocks were maintained in vials of sterile water at 4º C.

Antifungal Drugs and Preparation

AMB and ITC were purchased from Sigma and Research Diagnostics Inc., respectively, while VRC was generously provided by Pfizer (UK). Stock concentrations of 1600 µg/ml were prepared for each drug with dimethyl sulfoxide (DMSO) and stored at -80º C for up to six months. For mRSA testing, serial two-fold dilution series of AMB, ITC, and VRC were prepared in DMSO to achieve drug concentration ranges of 3 to 1600 µg/ml. Each DMSO dilution was then diluted 1:50 in 0.4% glucose RPMI to achieve 2x drug concentration ranges of 0.06 to 32 µg/ml. Both drug-free growth and glucose controls were prepared, containing the same ratio of DMSO to 0.4% glucose RPMI as the 2x drug dilutions.

Microwell Plate Preparation

Testing of non-*A. fumigatus* molds was performed in 96 well round bottom plates. Test wells for each mold were prepared by inoculating 100 µl of 32 µg/ml AMB, 32 µg/ml ITC, and drug-free 0.4% glucose RPMI (growth control), containing the same concentration of DMSO as RPMI containing drug, into triplicate wells. 100 µl of drug-free 0.4 % glucose RPMI was also added to an additional three wells to represent the glucose control (no drug or mold). Plates were prepared on the day of use and stored at 4ºC until needed.

mRSA and NCCLS M38-A testing was performed in duplicate in 96 well round bottom plates with each row, comprised of 12 wells, corresponding to one susceptibility test. Wells 1-10 were inoculated with 100 µl of the 2 x AMB, ITC, or VRC dilution ranges (0.06 to 32 µg/ml). Wells 11 and 12, the drug-free growth and glucose controls, respectively, were inoculated with 100 µl of DMSO - 0.4% glucose RPMI. Plates were prepared on the day of use and stored at 4º C until needed.

Inoculum Preparation

A. fumigatus and non-*A. fumigatus* isolates were cultured on SAB agar slants at 30º C for three or four days, respectively. The slant surface was flooded with glucose deficient RPMI and gently scraped with a sterile wooden applicator stick. Once the hyphal fragments were allowed to settle for 15 min, the conidial suspension was collected and adjusted with glucose deficient RPMI to an optical density (OD_{530nm}) of 0.09 to 0.11 for non-*A. fumigatus* spp. and 0.11 for *A. fumigatus* isolates. If necessary, conidial suspensions were further diluted 1:50 with glucose deficient RPMI. A conidial suspension could not be obtained with *F. oxysporum* FO1. Testing of this strain was instead performed with a 0.13 OD_{530nm} hyphal inoculum. Conidia were enumerated microscopically using a hemocytometer and/or by CFU/ml counts.

Glucose Utilization of Conidial or Hyphal Inocula of Non-*A. fumigatus* Molds in Presence or Absence of AMB and ITC

Testing of *A. flavus*, *A. sydowii*, *A. terreus* and *S. angiospermum* strains was initiated by inoculating 100 µl of each strains' 0.09-0.11 OD_{530nm} conidial inocula (except

F. oxysporum FO1) into wells containing 100 µl of AMB, ITC, or drug-free RPMI. Testing of *F. oxysporum* was initiated by inoculating 100 µl of the 0.13 OD_{530nm} hyphal inoculum into wells containing 100 µl of AMB, ITC, or drug-free RPMI. 100 µl of glucose deficient RPMI was added to glucose control wells. Plates were sealed with tape and placed into a 35-37º C incubator. Four identical sets of plates were prepared and placed into incubator at time 0, permitting one set to be used for determination of glucose utilization and antifungal effect at 5, 18.5, 24, and 48 h.

The glucose utilization of conidial and hyphal inocula was determined at each time point by adding 50 µl of color mix testing wells. After 20 min, the optical density ($OD_{550\text{-}655nm}$) in each well was determined by spectrophotometer. Percent residual glucose values were calculated by comparing the $OD_{550\text{-}655nm}$ value of each AMB, ITC, or growth control test well with the $OD_{550\text{-}655nm}$ of the glucose control well based on the following equation: ($OD_{550\text{-}655nm}$ of test well / $OD_{550\text{-}655nm}$ of glucose control well) X 100.

After residual glucose levels had been determined, a pipette tip was used to scrape the bottom and sides of each well and transfer approximately 50 µl of the mixture to a glass slide. After placement of a coverslip, the slides were observed by light microscopy at 400X. Antifungal effect on conidia or hyphae was determined by comparing the conidial or hyphal appearance in the presence and absence of 16 µg/ml of AMB or ITC.

mRSA Assay and MIC Determination

The mRSA was initiated by inoculating wells 1-11 with 100 µl of the 0.11 OD_{530nm} or 1:50 0.11 OD_{530nm} conidial inoculum of each *A. fumigatus* or *A. flavus* test strain, well 12 with 100 µl of glucose deficient RPMI, sealing the plate edges with tape,

and incubation at 35-37º C. The plates were removed from the incubator after approximately 16 h and residual glucose levels determined by addition of 50 µl of an enzyme color mix comprised of 0.6 M sodium phosphate buffer (pH 6.0), 360 µg/ml of 4-amino antipyrine, 490 µg/ml of N-ethyl-N-sulfopropyl-m-toluidine, 0.68 U/ml horseradish peroxidase, and 0.4 U/ml glucose oxidase to each well. At 20 min, the color intensity was measured at OD_{550nm} with a reference reading of OD_{655nm} in a plate reader. Percent residual glucose values were calculated by comparing the $OD_{550\text{-}655nm}$ value of each AMB, ITC, VRC, or growth control test well with the $OD_{550\text{-}655nm}$ of the glucose control well based on the following equation: ($OD_{550\text{-}655nm}$ of test well / $OD_{550\text{-}655nm}$ of glucose control well) X 100.

Antifungal susceptibility curves were generated for each mRSA test by plotting the percent residual glucose values (y-axis) against the appropriate AMB, ITC, or VRC concentrations (x-axis). The resulting detection interval contained an upper and lower limit represented, respectively, by the percent residual values corresponding to the highest concentration of antifungal drug and the drug-free growth control. mRSA AMB MICs were defined as the lowest drug concentration having a percent residual glucose value within the top 10% of the detection interval. mRSA ITC and VRC MICs were defined as the lowest drug concentration corresponding to a residual glucose value within the top 20% of the detection only if a) a detection interval comprised of an upper plateau, steep decline, and lower plateau was discernable and b) the highest concentration of ITC or VRC (16 µg/ml) had a corresponding residual glucose value of ≥ 80%. If these conditions were not met, the ITC or VRC MIC_{RSA} was designated as >16 µg/ml.

NCCLS M38-A Testing and MIC Determination

The NCCLS M38-A assay was initiated by inoculating wells 1-11 with 100 µl of the 1:50 0.11 OD_{530nm} conidial inoculum of each strain, well 12 with 100 µl of glucose deficient RPMI, sealing the plate edges with tape, and incubation at 35-37º C. NCCLS M38-A MICs were determined by visual inspection at 48 h using a mirror plate reader according the NCCLS M38-A guidelines (142) with AMB, ITC, and VRC MIC endpoint values designated as the lowest drug concentration preventing growth.

Preliminary mRSA Testing of Non-*A. fumigatus* Mold Species Results and Discussion

The effects of 16 µg/ml AMB and ITC on both the conidial development and glucose utilization of *A. flavus*, *A. sydowii*, *A. terreus*, *S. angiospermum* 0.11 OD_{530nm} conidial inocula are shown in Figures 20 thru 35. Like *A. fumigatus*, *A. flavus* typically has both in vitro and in vivo susceptibility to AMB and ITC (246), although both AMB and ITC resistance has been reported (139, 246). In the absence of drug, *A. flavus* AFL1, AFL2, and AFL3 conidia (Fig. 20A, 22A, 24A) germinated in 5 h (data not shown), with mature hyphae (hyphae greater than three times conidial diameter width) observed after 18.5 h (Fig. 20B, 22B, 24B). This corresponded to a rate of rapid utilization of glucose between 0 and 24 h followed by a slower rate between 24 and 48 h (Fig. 21, 23, 25). In the presence of 16 µg/ml AMB, conidial development was inhibited prior to germination (Fig. 20C, 22C, 24C). In the presence of 16 µg/ml ITC, conidial development was inhibited either prior to (Fig. 20D, 22D) or immediately after germination (Fig. 24D).

AMB and ITC also caused inhibition of glucose utilization between 0 and 5 h (Fig. 21, 23, 25). These data indicate a direct correlation between the effects of AMB and ITC on both conidial development and glucose utilization. As the 24 h glucose utilization of *A. flavus* strains (70 to 80%) was greater than that of *A. fumigatus* strains (~60%), it may be

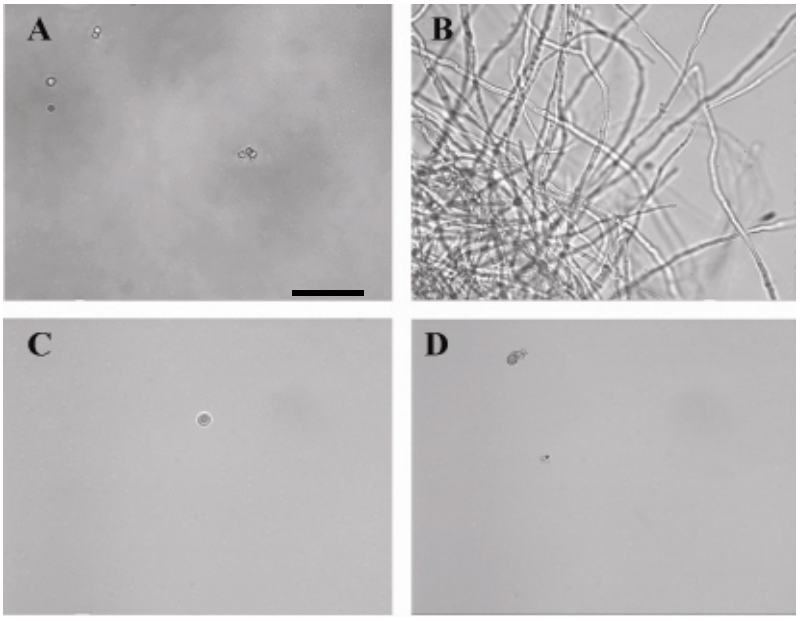


Figure 20. *A. flavus* AFL1 conidial development before and after 48 h incubation at 35-37º C in the presence or absence of AMB or ITC. Wet-mount slide preps observed (X400) at 0 h (A) and 48 h (B) with no antifungal agent; 48 h with 16 µg/ml AMB (C); and 48 h with 16 µg/ml ITC (D). Bar represent 25 µm.

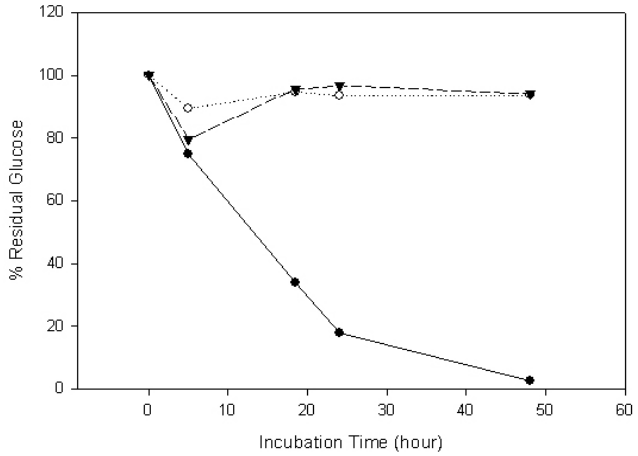


Figure 21. Glucose utilization by *A. flavus* AFL1 0.11 OD_{530nm} conidial inoculum during 48 h incubation at 35-37º C in the presence of no antifungal drug (●), 16 µg/ml AMB (○), or 16 µg/ml ITC (▼).

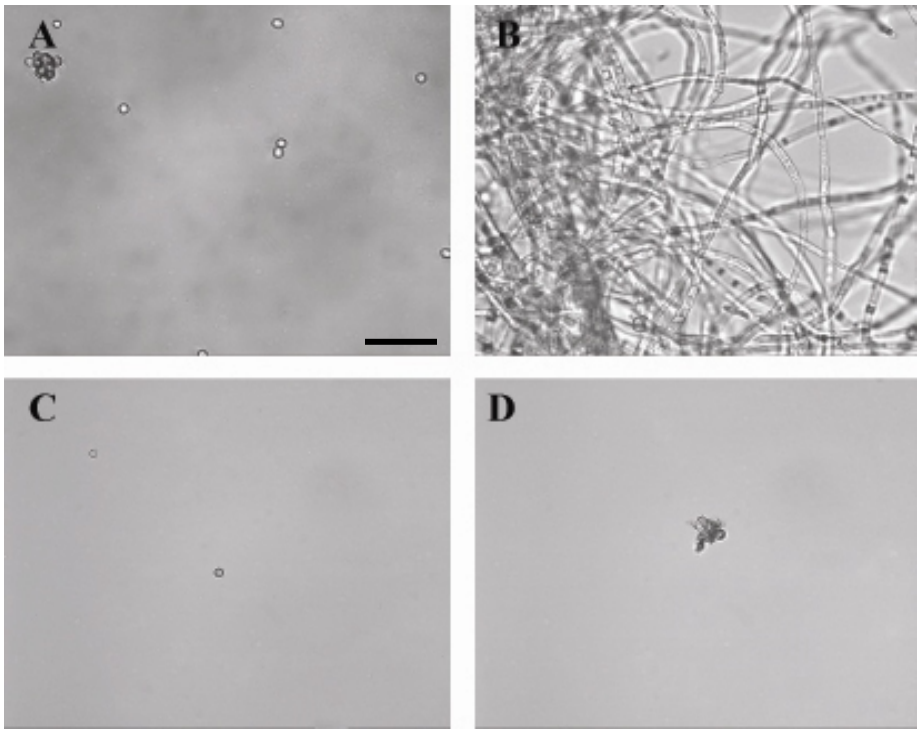


Figure 22. *A. flavus* AFL2 conidial development before and after 48 h incubation at 35-37º C in the presence or absence of AMB or ITC. Wet-mount slide preps observed (X400) at 0 h (A) and 48 h (B) with no antifungal agent; 48 h with 16 µg/ml AMB (C); and 48 h with 16 µg/ml ITC (D). Bar represents 25 µm.

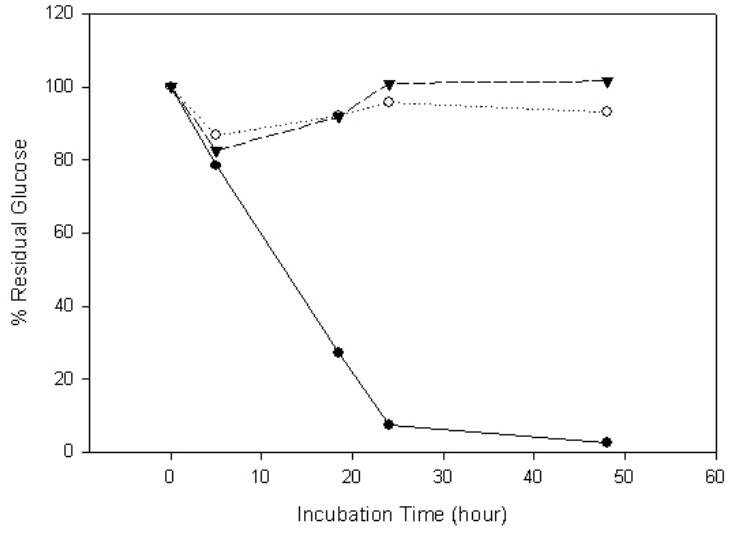


Figure 22. Glucose utilization by *A. flavus* AFL2 0.11 OD_{530nm} conidial inoculum during 48 h incubation at 35-37º C in the presence of no antifungal drug (●), 16 µg/ml AMB (○), or 16 µg/ml ITC (▼).

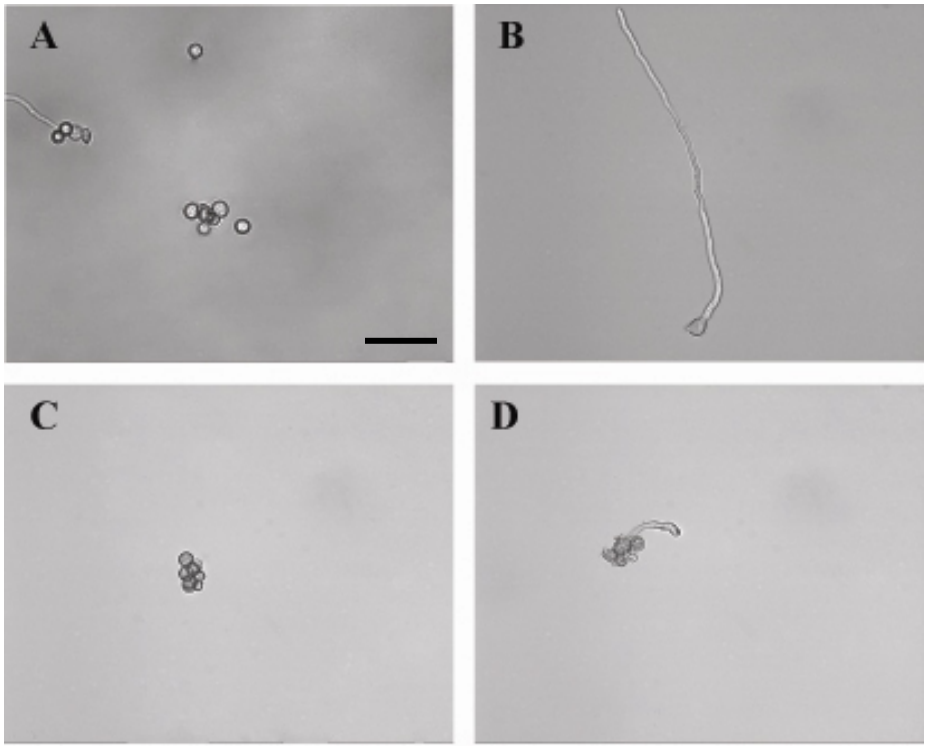


Figure 24. *A. flavus* AFL3 conidial development before and after 48 h incubation at 35-37º C in the presence or absence of AMB or ITC. Wet-mount slide preps observed (X400) at 0 h (A) and 48 h (B) with no antifungal agent; 48 h with 16 µg/ml AMB (C); and 48 h with 16 µg/ml ITC (D). Bar represents 25 µm.

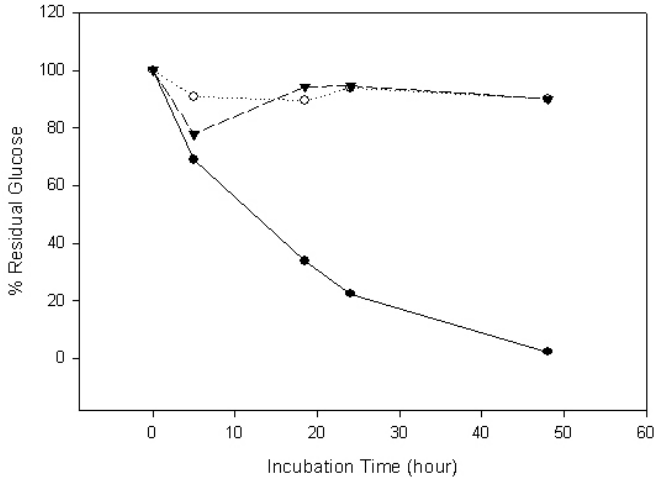


Figure 25. Glucose utilization by *A. flavus* AFL3 0.11 OD_{530nm} conidial inoculum during 48 h incubation at 35-37º C in the presence of no antifungal drug (●), 16 µg/ml AMB (○), or 16 µg/ml ITC (▼).

feasible to perform *A. flavus* mRSA testing in less than the 16 h required for *A. fumigatus*.

Although rare, *A. sydowii* can be an etiologic agent of aspergillosis (33). While no information was found regarding the AMB sensitivity of *A. sydowii*, it has been reported to be sensitive to ITC (247). In the absence of drug, the conidia of *A. sydowii* strains AS1 and AS2 (Fig. 26A, 28A) germinated during the interval between 5 and 18.5 h (data not shown), with mature hyphae (hyphae greater than three times conidial diameter width) observed after 18.5 h (Fig. 26B, 28B). The corresponding glucose utilization pattern of each conidial inoculum during the 48 h incubation was different between strains. *A. sydowii* AS1 demonstrated a low rate of glucose utilization between 0 and 18.5 h, which rapidly increased between 18.5 and 24 h, before returning to its original rate for the remainder of the incubation (Fig. 27). In contrast, the rate of glucose utilization with *A. sydowii* AS2 was fairly constant during the 48 h incubation period (Fig. 29). Both AMB and ITC inhibited conidial development prior to germination (Fig. 26C, 28C), which corresponded to residual glucose levels indicating inhibition of glucose utilization by AMB and ITC prior to 18.5 h (Fig. 27, 29). These data suggest a direct correlation between the effects of AMB and ITC on *A. sydowii* conidial development and the conidia's subsequent glucose utilization. Because the 24 h glucose utilization of *A. sydowii* conidial inocula (25-40%) was less than the 24 h glucose utilization of *A. fumigatus* conidial inocula (60%), it may be necessary to use a 24 or 48 h mRSA incubation time for mRSA testing of *A. sydowii* strains.

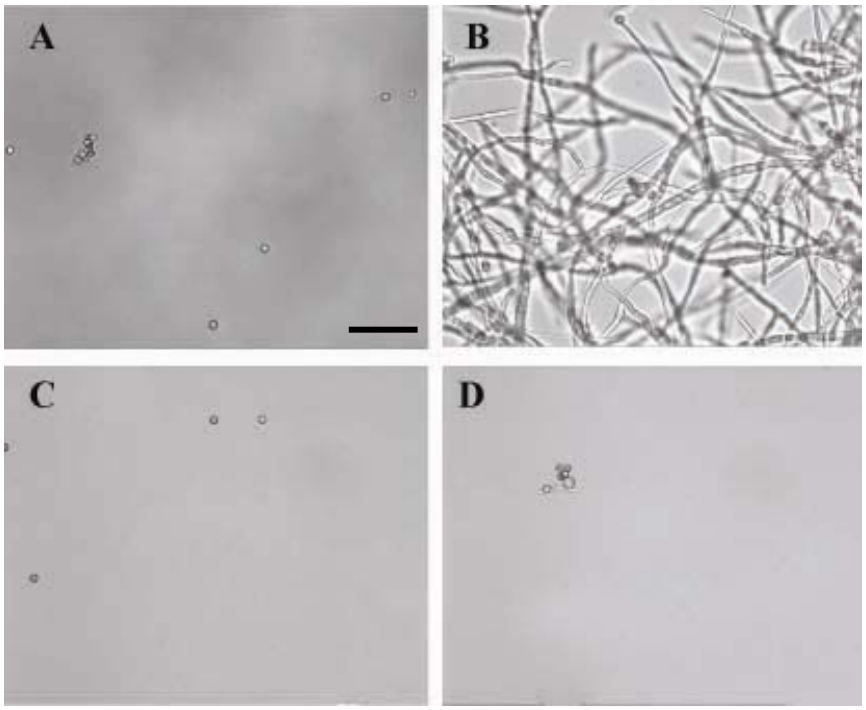


Figure 26. *A. sydowii* AS1 conidial development before and after 48 h incubation at 35-37ºC in the presence or absence of AMB or ITC. Wet-mount slide preps observed (X400) at 0 h (A) and 48 h (B) with no antifungal agent; 48 h with 16 µg/ml AMB (C); and 48 h with 16 µg/ml ITC (D). Bar represents 25 µm.

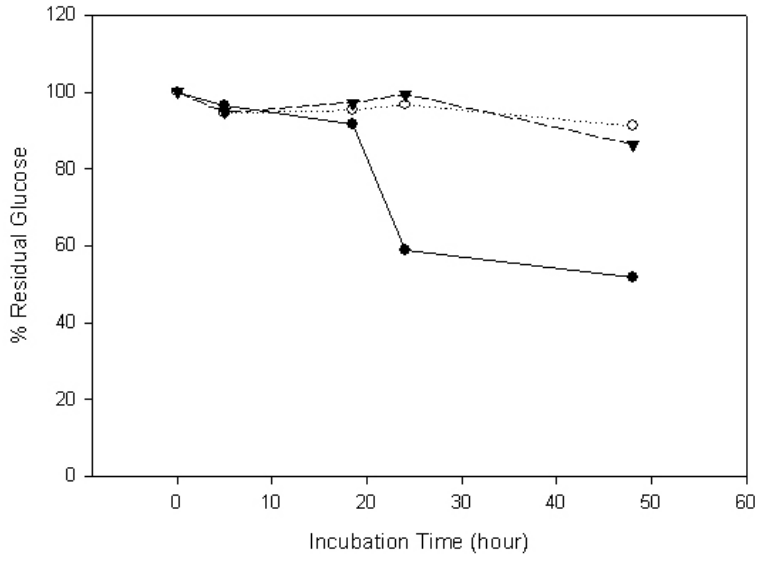


Figure 27. Glucose utilization by *A. sydowii* AS1 0.11 OD_{530nm} conidial inoculum during a 48 h incubation at 35-37ºC in the presence of no antifungal drug (●), 16 µg/ml AMB (○), or 16 µg/ml ITC (▼).

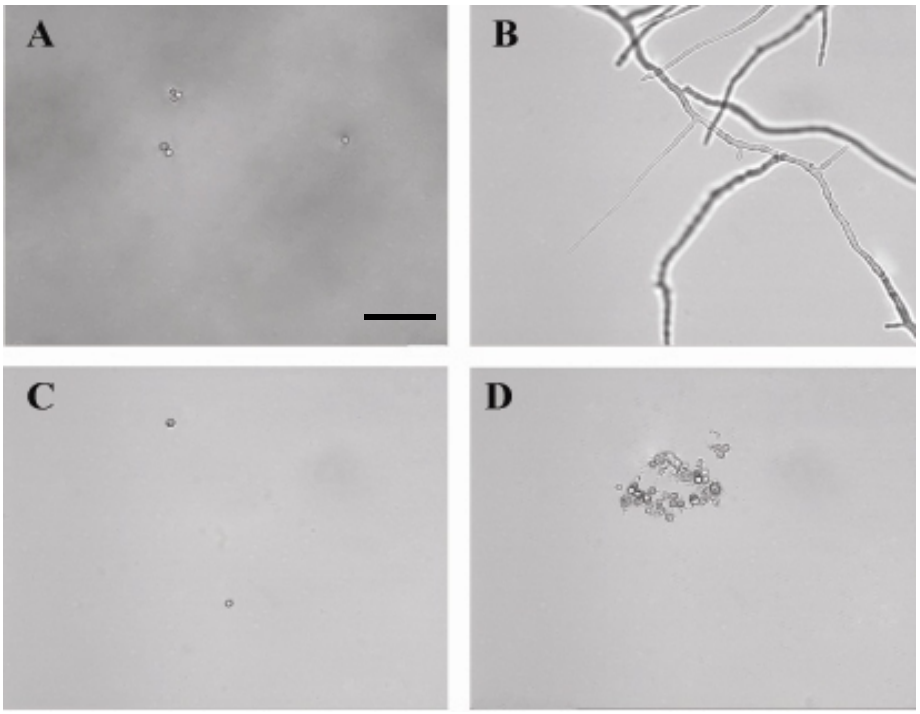


Figure 28. *A. sydowii* AS2 conidial development before and after 48 h incubation at 35-37º C in the presence or absence of AMB or ITC. Wet-mount slide preps observed (X400) at 0 h (A) and 48 h (B) with no antifungal agent; 48 h with 16 µg/ml AMB (C); and 48 h with 16 µg/ml ITC (D). Bar represents 25 µm.

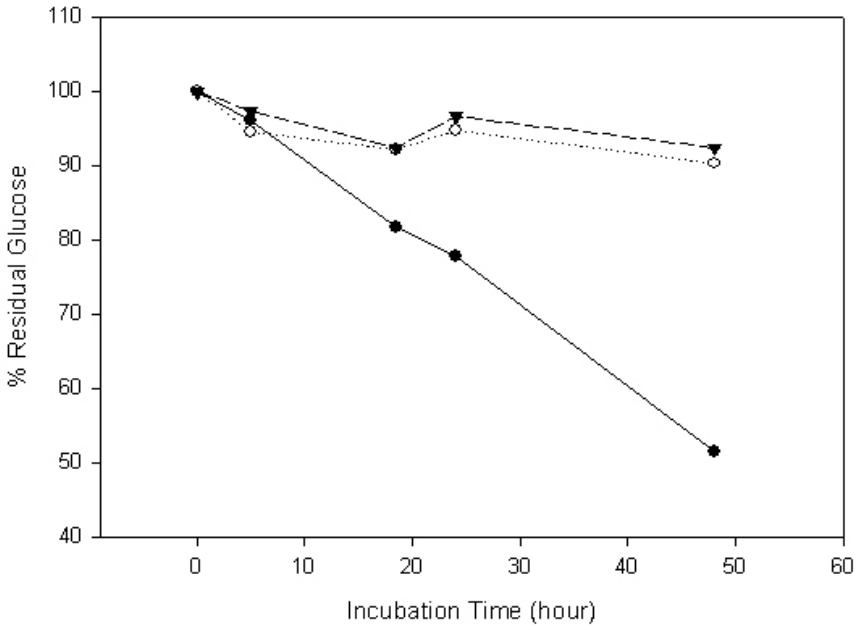


Figure 29. Glucose utilization by *A. sydowii* AS2 0.11 OD_{530nm} conidial inoculum during a 48 h incubation at 35-37º C in the presence of no antifungal drug (●), 16 µg/ml AMB (○), or 16 µg/ml ITC (▼).

The number of reports of pneumonia and disseminated infections caused by *A. terreus* is increasing. While *A. terreus* is reported to be sensitive to ITC both in vivo and in vitro (114), it has been reported to be resistant to AMB in vivo, with in vitro AMB MICs ranging from 2 to 4 μg/ml (114). In the absence of drug, *A. terreus* AT1 conidia (Fig. 30A) germinated between 5 and 18.5 h incubation (data not shown), with mature hyphae observed after 18.5 h (Fig. 30B). This corresponded to a moderate rate of glucose utilization between 0 and 18.5 h, a more rapid rate between 18.5 and 24 h, and another moderate rate of glucose utilization from 24 to 48 h (Fig. 31). Both AMB and ITC inhibited conidial development prior to germination (Fig. 30C, D). This corresponded to residual glucose levels (~92%) that indicated inhibition of glucose utilization by AMB and ITC prior to 18.5 h (Fig. 31). These data suggest a direct correlation between the effects of AMB and ITC on *A. terreus* conidial development and the conidia's subsequent glucose utilization. Because the 24 h glucose utilization of the *A. terreus* conidial inoculum (~65%) was similar to the 24 h glucose utilization *A. fumigatus* conidial inocula (~60%), I predict that *A. terreus* mRSA testing can be performed in 16 h. In preliminary *A. terreus* mRSA testing described in Chapter 2, I did in fact find that 16 h was sufficient to obtain mRSA AMB and ITC MICs that were equal to or within two-fold dilution of those obtained by the NCCLS M38-P assay in 48 h. However, the mRSA AMB MIC was 0.5 μg/ml after 16 h incubation, which indicated AMB sensitivity. Incubating for 48 h increased the mRSA AMB MIC to 4 μg/ml. If all *A. terreus* strains are resistant to AMB, perhaps a mRSA incubation time of 48 h may be more appropriate, even though the glucose utilization indicates that 16 h should be sufficient. Additional

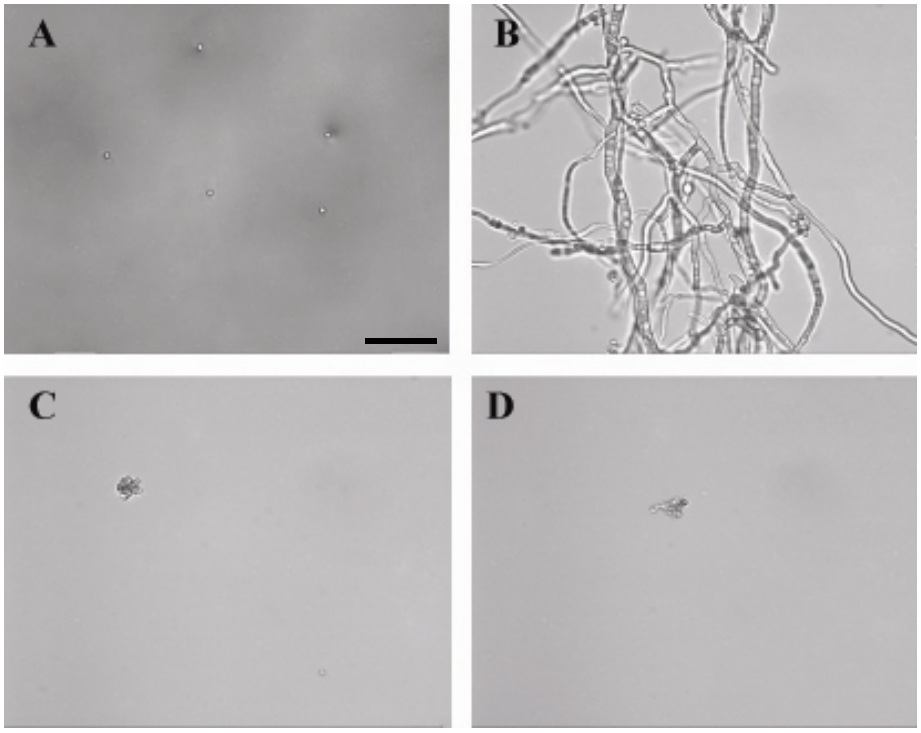


Figure 30. *A. terreus* AT1 conidial development before and after a 48 h incubation at 35-37º C in the presence or absence of AMB or ITC. Wet-mount slide preps observed (X400) at 0 h (A) and 48 h (B) with no antifungal agent; 48 h with 16 µg/ml AMB (C); and 48 h with 16 µg/ml ITC (D). Bar represents 25 µm.

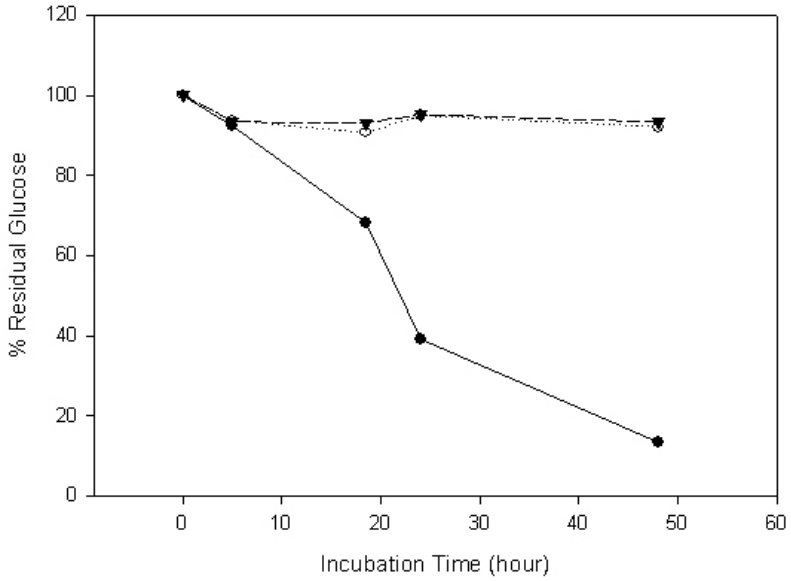


Figure 31. Glucose utilization by *A. terreus* AT1 0.11 OD_{530nm} conidial inoculum during 48 h incubation at 35-37º C in the presence of no antifungal drug (●), 16 µg/ml AMB (○), or 16 µg/ml ITC (▼).

mRSA testing of other *A. terreus* isolates, especially those isolated from patients with known clinical failure, is necessary to resolve this matter.

S. angiospermum (*Pseudallescheria boydii*), another rare but increasingly prevalent etiologic agent of hyalohyphomycosis, is resistant to AMB and ITC both in vitro and in vivo, with AMB and ITC MICs ranging from 0.25-16 and 0.25-4 μg/ml, respectively (139, 193, 241, 242, 248). In the absence of drug, *S. angiospermum* SA1 conidia (Fig. 32A) germinated between 24 and 48 h incubation (data not shown), with mature hyphae (hyphae greater than three times conidial diameter width) observed at 48 h (Fig. 32B). This corresponded to a relatively steady rate of glucose utilization throughout the 48 h incubation (Fig. 33). There is some question as to why so much glucose was being utilized before 24 h without germination occurring. Testing of additional *S. angiospermum* strains is required to determine if this observation is strain or species specific. Both AMB and ITC inhibited conidial development prior to germination (Fig. 32C, D). This corresponded to residual glucose levels (~95%) that indicated inhibition of glucose utilization by AMB and ITC prior to conidial germination (Fig. 33). These data suggest a direct correlation between the effects of AMB and ITC on *S. angiospermum* conidial development and the conidia's subsequent glucose utilization. As the 24 h glucose utilization of the *S. angiospermum* conidial inoculum (~40%) was less than the 24 h glucose utilization of *A. fumigatus* conidial inocula (~60%), it may be necessary to use a 24 or 48 h mRSA incubation time for mRSA testing of *S. angiospermum* strains. This was not unexpected as the NCCLS M38-A protocol currently recommends 70 to 74 h incubation for AMB and ITC testing of *S. angiospermum* isolates (128).

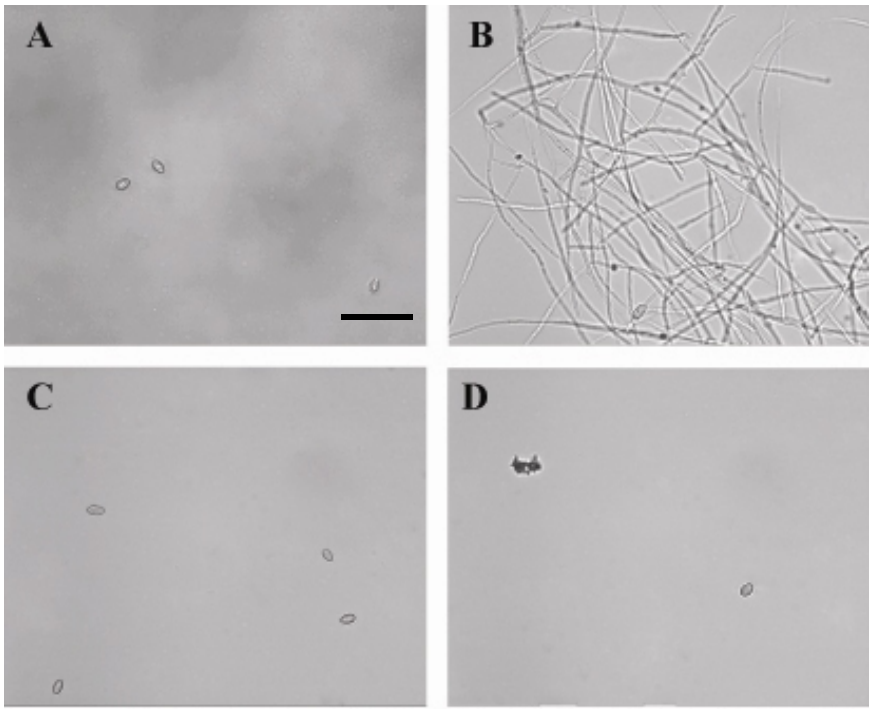


Figure 32. *S. angiospermum* SA1 conidial development before and after a 48 h incubation at 35-37º C in the presence or absence of AMB or ITC. Wet-mount slide preps observed (X400) at 0 h (A) and 48 h (B) with no antifungal agent; 48 h with 16 µg/ml AMB (C); and 48 h with 16 µg/ml ITC (D). Bar represents 25 µm.

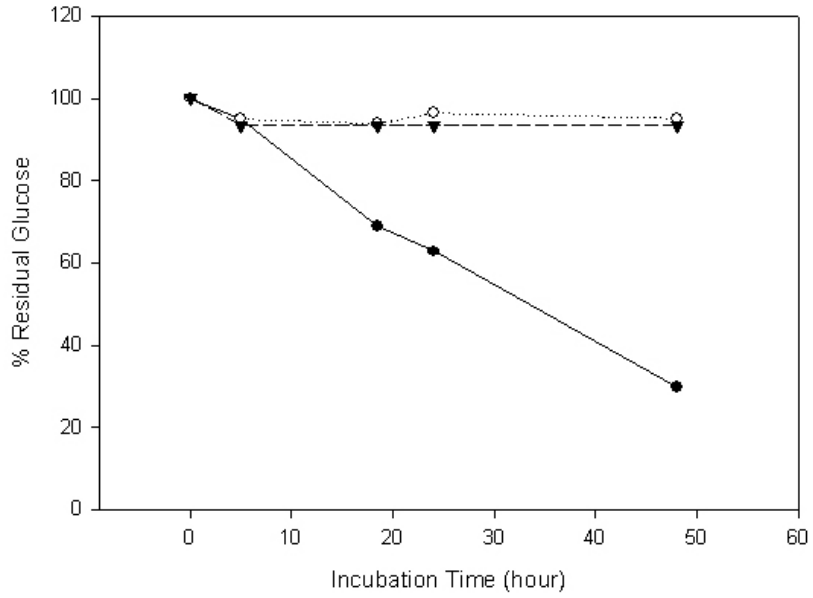


Figure 33. Glucose utilization by *S. angiospermum* SA1 0.11 OD_{530nm} conidial inoculum during 48 h incubation at 35-37º C in the presence of no antifungal drug (●), 16 µg/ml AMB (○), or 16 µg/ml ITC (▼).

Fusarium oxysporum is an emerging opportunistic fungal pathogen that can cause severe disseminated infections in neutropenic patients (244). *F. oxysporum* is resistant to AMB and ITC in vivo and usually in vitro, with AMB and ITC MICs ranging from 0.25 – >16 and 0.5– >16 μg/ml, respectively (139, 151, 241, 242, 249). The inability to obtain a conidial inoculum may have been due to the use of SAB agar as the growth medium. Perhaps potato dextrose agar should have been used as this medium induces greater conidial sporulation. As a conidial inoculum could not be prepared, I instead tested a 0.13 OD_{530nm} hyphal inoculum. The appearance of *F. oxysporum* hyphae before and after 48 h incubation in the absence of AMB and ITC are shown in Fig. 34A and B, respectively. There is no discernable difference in hyphal appearance between time points. This corresponded to an irregular rate of glucose utilization during the 48 h incubation. The rate of glucose utilization was moderate until 18.5 h, when a negative rate of utilization occurred until 24 h, followed by a very low rate from 24 to 48 h (Fig. 35). Hyphae incubated in the presence of 16 μg/ml AMB appeared fragmented by 48 h (Fig. 34C). In contrast, hyphae incubated in the presence of 16 μg/ml ITC for 48 h appeared normal (Fig. 34D). Residual glucose levels in the medium following 48 h exposure of hyphae to AMB and ITC ranged from 80 to 90%, indicating that the AMB and ITC effect on glucose utilization occurred within 10 h (Fig. 35). These data suggest a direct correlation between the effect of AMB on *F. oxysporum* hyphae and the hyphae's subsequent glucose utilization. However, establishment of a direct correlation with ITC effect on hyphal appearance and the hyphae's subsequent glucose utilization is more difficult. Although glucose utilization was inhibited in the presence of ITC, the hyphae

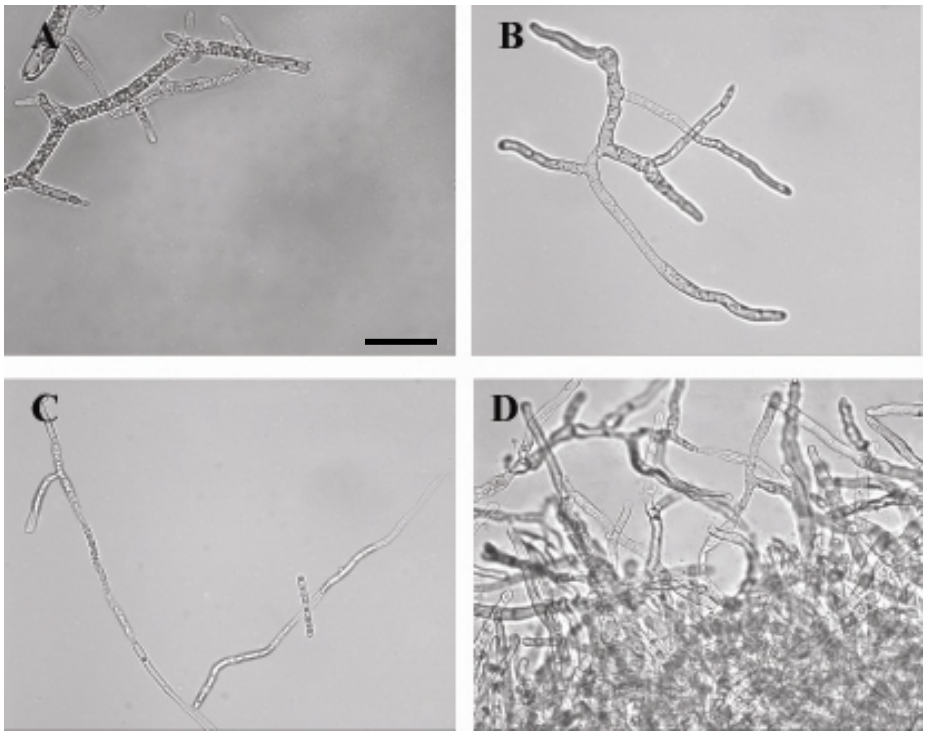


Figure 34. *F. oxysporum* FO1 conidial development before and after 48 h incubation at 35-37º C in the presence or absence of AMB or ITC. Wet-mount slide preps observed (X400) at 0 h (A) and 48 h (B) with no antifungal agent, 16 µg/ml AMB (C), and 48 h with 16 µg/ml ITC (D). Bar represents 25 µm.

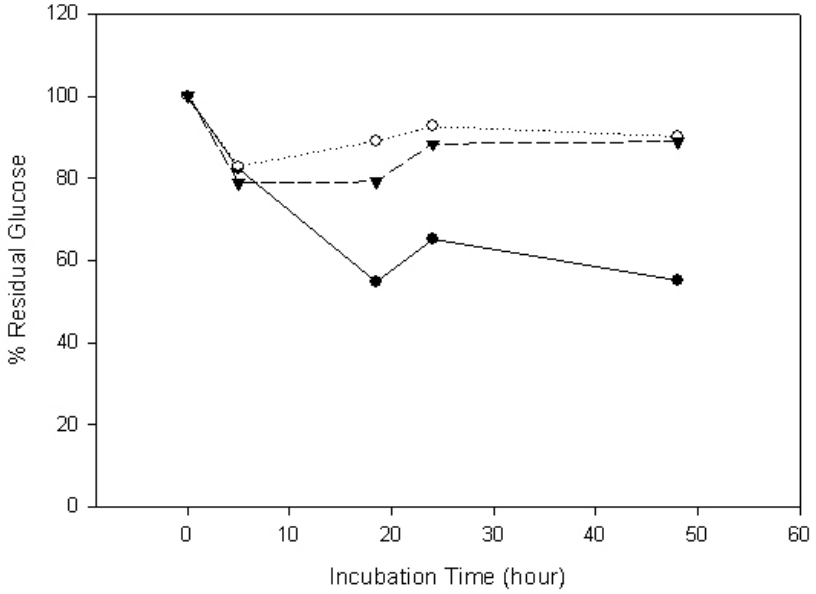


Figure 35. Glucose utilization by *F. oxysporum* FO1 0.13 OD_{530nm} hyphal inoculum during 48 h incubation at 35-37º C in the presence of no antifungal drug (●), 16 µg/ml AMB (○), or 16 µg/ml ITC (▼).

appeared normal. As *F. oxysporum* usually produces ITC MICs of >8 µg/ml, one possibility is that the ITC concentration of 16 µg/ml was not concentrated enough to be fungicidal, but was instead fungistatic, inhibiting further hyphal growth and glucose utilization without affecting hyphal appearance.

I have demonstrated a direct correlation between the AMB and ITC effect on conidial development and the subsequent glucose utilization of *A. flavus*, *A. sydowii*, *A. terreus*, and *S. angiospermum* 0.11 OD_{530nm} conidial inocula. Now that these strains have been deemed suitable for mRSA testing, the next step is to optimize the mRSA testing conditions of inoculum concentration and incubation temperature. mRSA testing of the *F. oxysporum* hyphal inoculum was also important as it demonstrated that a hyphal inoculum is suitable for AMB mRSA testing. Testing of additional *F. oxysporum* strains, especially those with known clinical histories, would be useful in determining whether hyphae inocula are suitable for ITC mRSA testing.

Introduction of Voriconazole to mRSA Testing Results and Discussion

VRC Effect on *A. fumigatus* Conidial Germination and Glucose Utilization

The effect of VRC on the development and glucose utilization of the 0.11 OD_{530nm} and 1:50 0.11 OD_{530nm} conidial inocula of each *A. fumigatus* strain was determined by incubating conidial inocula at 35º C for 16 h in the presence or absence of 16 µg/ml VRC. VRC had the same effect on all conidial inocula, with the 0.11 OD_{530nm} conidial inocula of *A. fumigatus* strain ATCC 204305 shown in Figure 36. VRC inhibited

conidial development after 3 to 6 h, in a swollen morphology similar to that seen in the presence of inhibitory concentrations of ITC. The rapid inhibition of conidial germination by VRC correlated with its rapid inhibitory effect on conidial glucose utilization, which occurred within the first few hours of incubation (Figure 37).

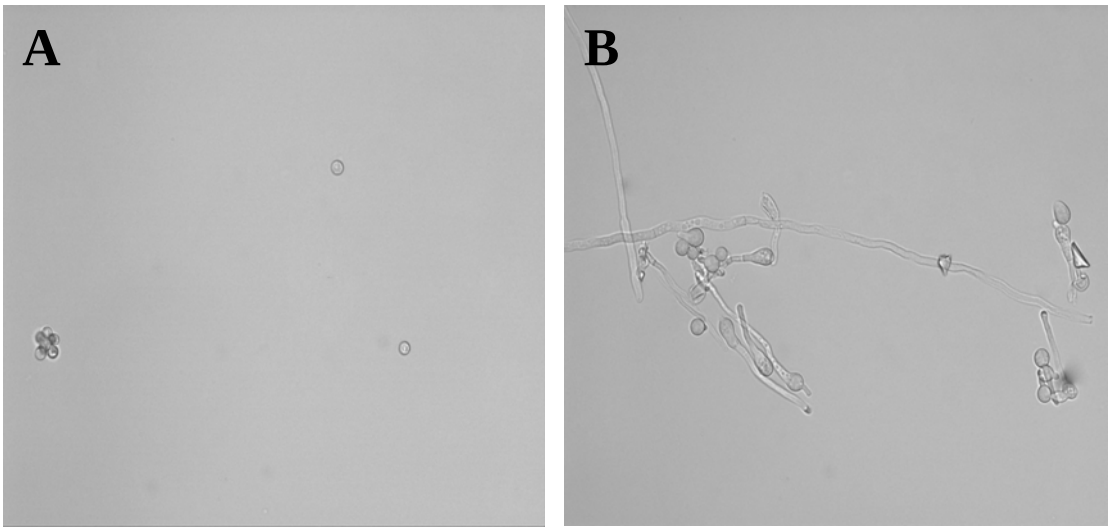


Figure 36. Appearance of ATCC 204305 conidia at 400x after a 16 h, 35º C incubation in the presence (A) or absence (B) of 16 µg/ml VRC. Bar indicates 25 µm.

Optimization of VRC Conditions in mRSA Testing

Optimal incubation time and conidial inoculum density conditions were defined as those providing a mRSA VRC MIC equal to or within a single two-fold dilution of the NCCLS M38-A VRC MIC within the shortest period of time. To determine whether current *A. fumigatus* AMB and ITC mRSA testing conditions would also be optimal in *A. fumigatus* mRSA VRC testing, I performed mRSA VRC testing in 16 h using 0.11 OD_{530nm} conidial inocula. mRSA VRC MICs were then compared to published and in-

house VRC MICs obtained by 48 h NCCLS M38-A testing (250) (Table 13). Like mRSA ITC susceptibility curves, VRC susceptibility curves were not as defined as those observed during AMB testing (data not shown), as an increased number of data points were located in the dose-dependent region of the curve. However, like the AMB and ITC susceptibility curves, all residual glucose values corresponding to inhibitory concentrations of VRC were >80%. The ITC MIC endpoint definition was also found to be optimal for VRC mRSA testing as the use of a 20%, not 10%, detection interval cutoff resulted in 16 h mRSA VRC MICs that were equal to or within one two-fold dilution of 48 h NCCLS M38-A VRC MICs.

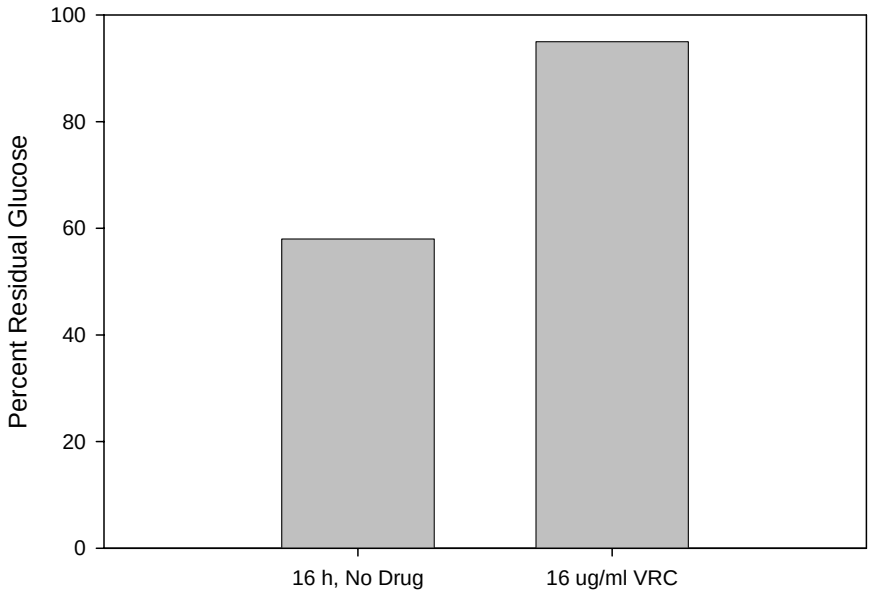


Figure 37. Percent residual glucose levels of 0.11 OD_{530nm} conidial inoculum of *A. fumigatus* strain ATCC 204305 after 16 h incubation in the presence or absence of 16 µg/ml VRC.

The major weakness of this work is the criterion used in determining optimal mRSA VRC testing conditions. Ensuring that 16 h mRSA VRC MICs are within one two-fold dilution of NCCLS M38-A VRC MICs is not a guarantee for clinically relevant mRSA MICs. However, VRC was only recently approved for wide scale use and the only VRC-resistant *A. fumigatus* strains currently available are laboratory-generated (251, 252). Therefore, as it was not possible to obtain VRC-resistant *A. fumigatus* clinical isolates for mRSA VRC optimization testing, our criterion was the most appropriate available. In addition, both the mRSA and NCCLS M38-A assays are capable of detecting ITC resistance, which increases the likelihood that current VRC mRSA conditions may also detect VRC resistance. Once VRC-resistant *A. fumigatus* strains become available, I recommend that they be tested in the mRSA to ensure that testing conditions are optimized for detecting VRC resistance.

Table 13. 16 h mRSA and 48 h NCCLS M38-A VRC MICs of *A. fumigatus* strains ATCC 204305, NCPF 7101, and NCPF 7100[a]

A. fumigatus Strain	Mold RSA VRC MIC	NCCLS M38-A VRC MIC	Published NCCLS M38-A VRC MIC[b]
ATCC 7100	2.0 μg/ml	1.0 μg/ml	1.0 μg/ml
NCPF 7101	0.25	0.5	0.5-1
ATCC 204305	0.25	0.5	NP[c]

[a] VRC MICs shown represent geometric mean of triplicate testing, with each test performed in duplicate.
[b] NCCLS M38-A VRC MIC values were previously published by Espinel-Ingroff et al. (65).
[c] NP, not published.

Validation of mRSA ITC Testing Results and Discussion

The mRSA and NCCLS M38-A AMB, ITC, and VRC MICs of new *A. fumigatus* clinical control strains are shown in Table 14. The mRSA and NCCLS M38-A AMB MICs of the AMB-sensitive strains CM237 and CM1369 indicated AMB sensitivity. However, the mRSA AMB MIC of CM1369 was four-fold less (0.125 µg/ml) than the NCCLS M38-A MIC (0.5 µg/ml). In addition, the mRSA AMB MIC of AF90 (0.25 µg/ml) was also four-fold less than the NCCLS M38-A AMB MIC (1 µg/ml) The mRSA

Table 14. RSA and NCCLS M38-A MICs of new *A. fumigatus* clinical strains

Strain ID	In vivo susceptibility	mRSA MIC (µg/ml)			NCCLS MIC (µg/ml)		
		AMB	ITC	VRC	AMB	ITC	VRC
CM237[a]	AMB – S[c]	0.25	0.25	0.25	0.5	0.25	0.25
CM1369		0.125	0.25	0.25	0.5	0.25	0.25
BR181[b]	ITC – R	0.25	8	0.13	0.5	>16	0.25
AF90		0.25	>16	0.5	1	>16	1
AF1422		0.5	>16	0.5	1	>16	1
AF72		1.0	>16	NT[d]	NT	NT	NT

[a] Strains donated by E. Mellado.
[b] Strains donated by D. Denning.
[c] S, sensitive. R, resistant.
[d] NT, not tested.

and NCCLS M38-A ITC MICs of the ITC-resistant strains BR181, AF90, AF1422, and AF72 indicated ITC resistance. However, the mRSA ITC of BR181 (8 µg/ml) was four-fold less than the NCCLS M38-A ITC MIC (>16 µg/ml). All mRSA VRC MICs were with one two-fold dilution of NCCLS M38-A VRC MICs.

mRSA and NCCLS M38-A testing of the new AMB-sensitive and ITC-resistant *A. fumigatus* isolates validated that mRSA conditions were optimized to detect AMB

sensitivity and ITC resistance. However, not all mRSA MICs were within a two-fold dilution of NCCLS M38-A MICs. Although these mRSA MICs were four-fold greater than their corresponding NCCLS M38-A MICs, this difference did not change the MIC interpretation. For instance, AMB MICs of 0.25 and 1 μg/ml both indicate AMB sensitivity (i.e. within achievable AMB serum levels) while ITC MICs of 8 and >16 indicate ITC resistance (i.e. greater than achievable ITC serum levels). Based on these data it is perhaps better to say that optimal mRSA AMB and ITC conditions are those producing AMB and ITC MICs equal to or within two, two-fold dilutions of NCCLS M38-A AMB and ITC MICs. mRSA testing of additional *A. fumigatus* clinical strains may help resolve this issue.

Optimization of *A. flavus* AMB, ITC, and VRC mRSA Testing Conditions Results and Discussion

VRC Effect on Conidial Development and Glucose Utilization

As with AMB and ITC mRSA testing, the suitability of mRSA VRC testing was demonstrated by establishing a direct correlation between VRC effect on both conidial development and conidial glucose utilization. Incubation of 0.11 OD_{530nm} and 1:50 0.11 OD_{530nm} *A. flavus* conidial inocula in the presence of 16 μg/ml VRC resulted in inhibition of conidial development at approximately 5 h, immediately following conidial germination (Fig. 38A). Inhibition of conidial development also corresponded to inhibition of glucose utilization prior to 5 h, with 0.11 OD_{530nm} conidial inocula demonstrating greater levels of glucose utilization in the presence and absence of VRC

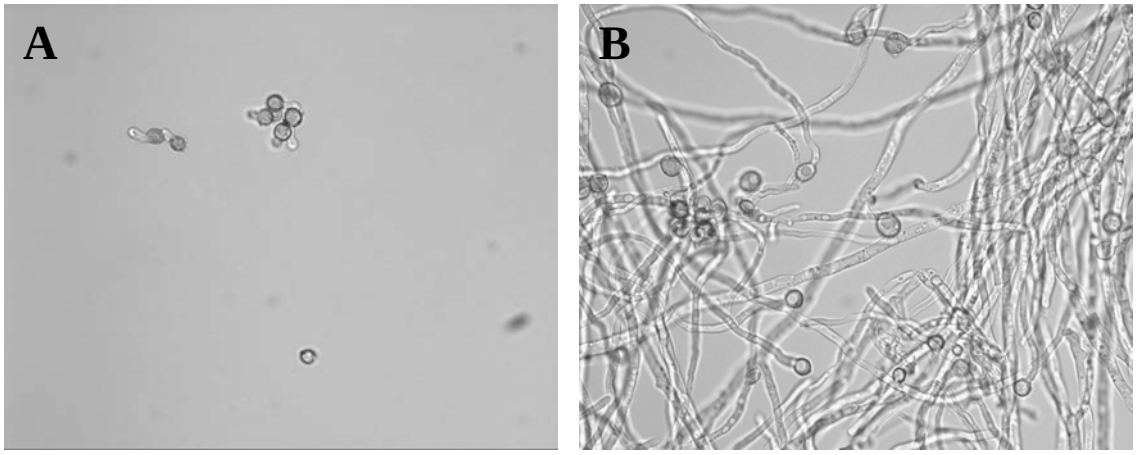


Figure 38. *A. flavus* conidial development after 16 h in the presence (A) or absence (B) of 16 µg/ml VRC.

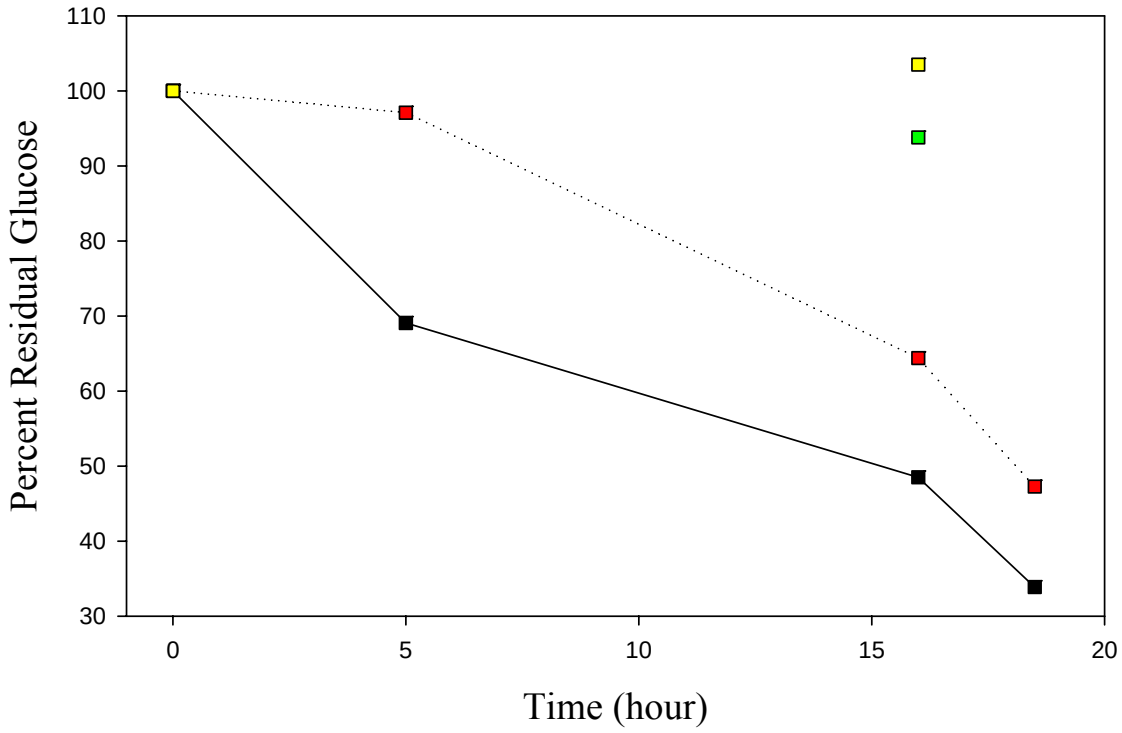


Figure 39. Glucose utilization of *A. flavus* 0.11 OD_{530nm} (■,■) and 1:50 0.11 OD_{530nm} (■,■) conidial inocula during 16 h incubation in the presence (■,■) or absence (■,■) of 16 µg/ml VRC.

compared to 1:50 0.11 OD_{530nm} conidial inocula (Fig. 39). Inhibition of conidial development and glucose utilization were similar in all *A. flavus* strains tested (data not shown).

mRSA AMB, ITC, and VRC Susceptibility Curves

The AMB, ITC, and VRC mRSA susceptibility curves corresponding to 0.11 OD_{530nm} and 1:50 0.11 OD_{530nm} conidial inocula of *A. flavus* strains AFL1, AFL2, and AFL3 are shown in Figures 40-42. In general, susceptibility curves corresponding to 1:50 0.11 OD_{530nm} conidial inocula appeared to be shifted to the right by two dilutions and slightly higher than the corresponding 0.11 OD_{530nm} susceptibility curves. With the exception of the *A. flavus* AFL1 1:50 0.11 OD_{530nm} susceptibility curve (Fig. 40A), the 0.11 OD_{530nm} and 1:50 0.11 OD_{530nm}, AMB susceptibility curves of each *A. flavus* strain were well defined with discernable upper plateaus, dose-dependent declines, and lower plateaus (Fig. 40A, 41A, 42A). The ITC (Fig. 40B, 41B, 42B) and VRC (Fig. 40C, 41C, 42C) susceptibility curves were not as defined as AMB susceptibility curves, with an increased number of data points in the dose-dependent decline and a small or absent lower plateau.

Comparison of mRSA and NCCLS M38-A AMB, ITC, and VRC MICs

The 16 h 0.11 OD_{530nm} and 1:50 0.11 OD_{530nm} conidial inocula mRSA and 48 h 1:50 0.11 OD_{530nm} conidial inocula NCCLS M38-A AMB, ITC, and VRC MICs are shown in Table 15. mRSA AMB were determined using an upper detection interval cutoff of 10%. mRSA ITC and VRC MICs were determined using an upper detection

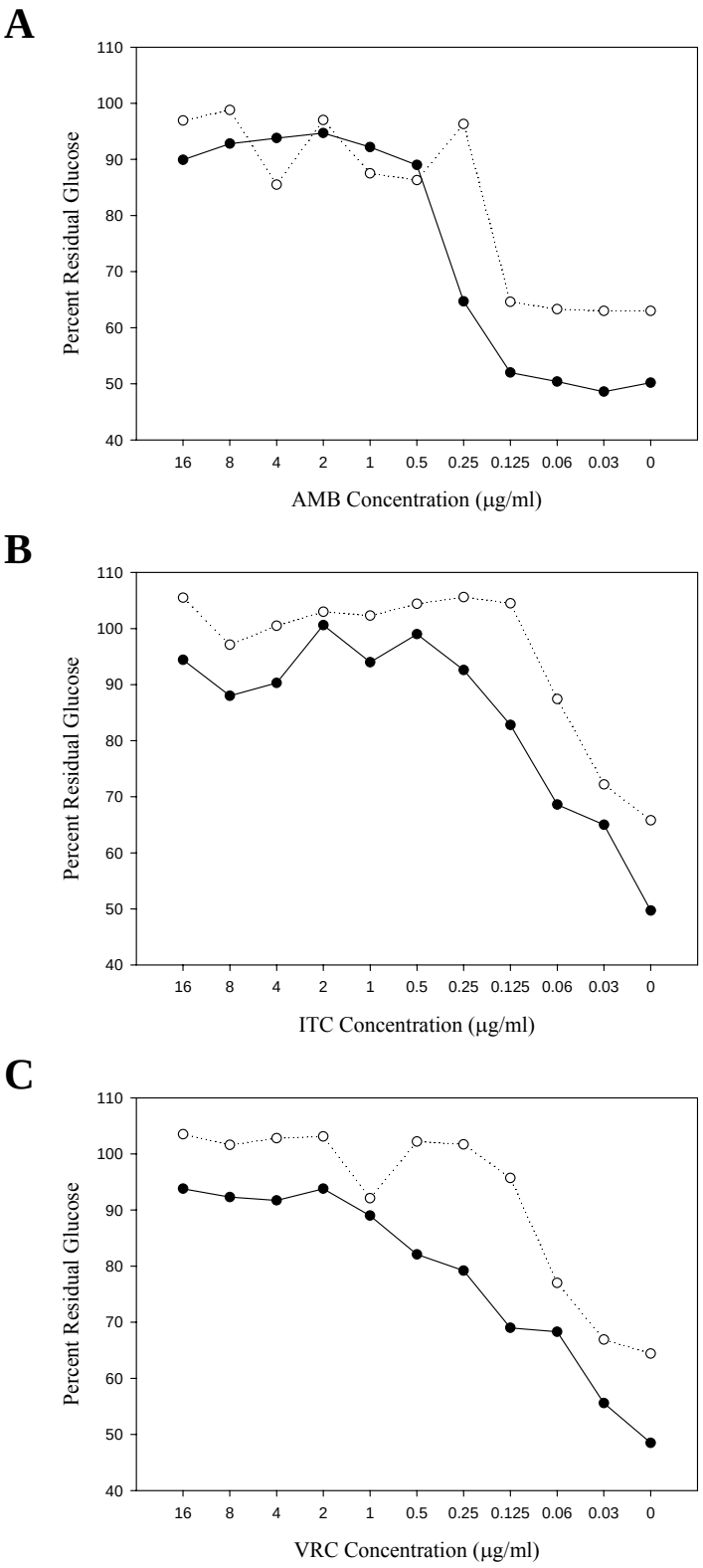


Figure 40. AMB (A), ITC (B), and VRC (C) mRSA susceptibility curves corresponding to 0.11 OD_{530nm} (●) and 1:50 0.11 OD_{530nm} (○) conidial inocula of *A. flavus* AFL1.

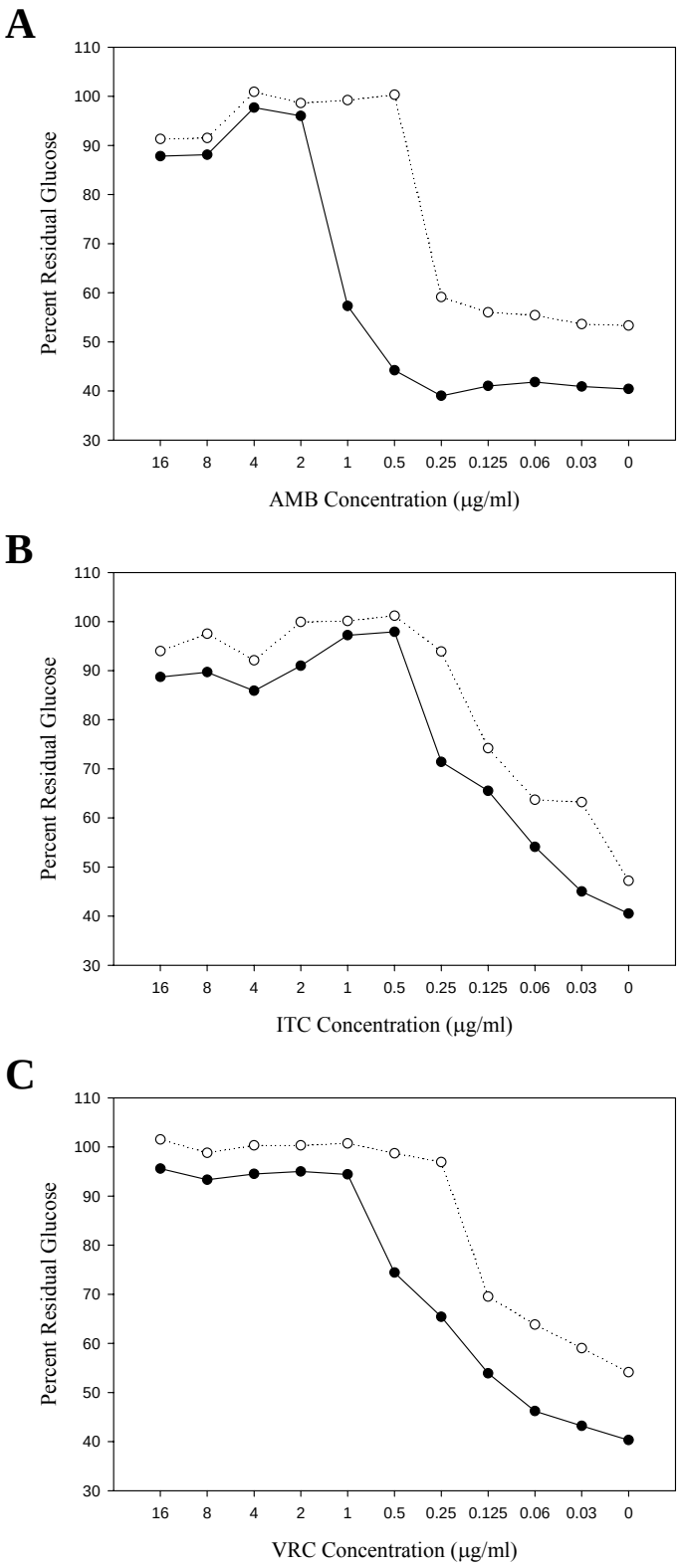


Figure 41. AMB (A), ITC (B), and VRC (C) mRSA susceptibility curves corresponding to 0.11 OD_{530nm} (●) and 1:50 0.11 OD_{530nm} (○) conidial inocula of *A. flavus* AFL2.

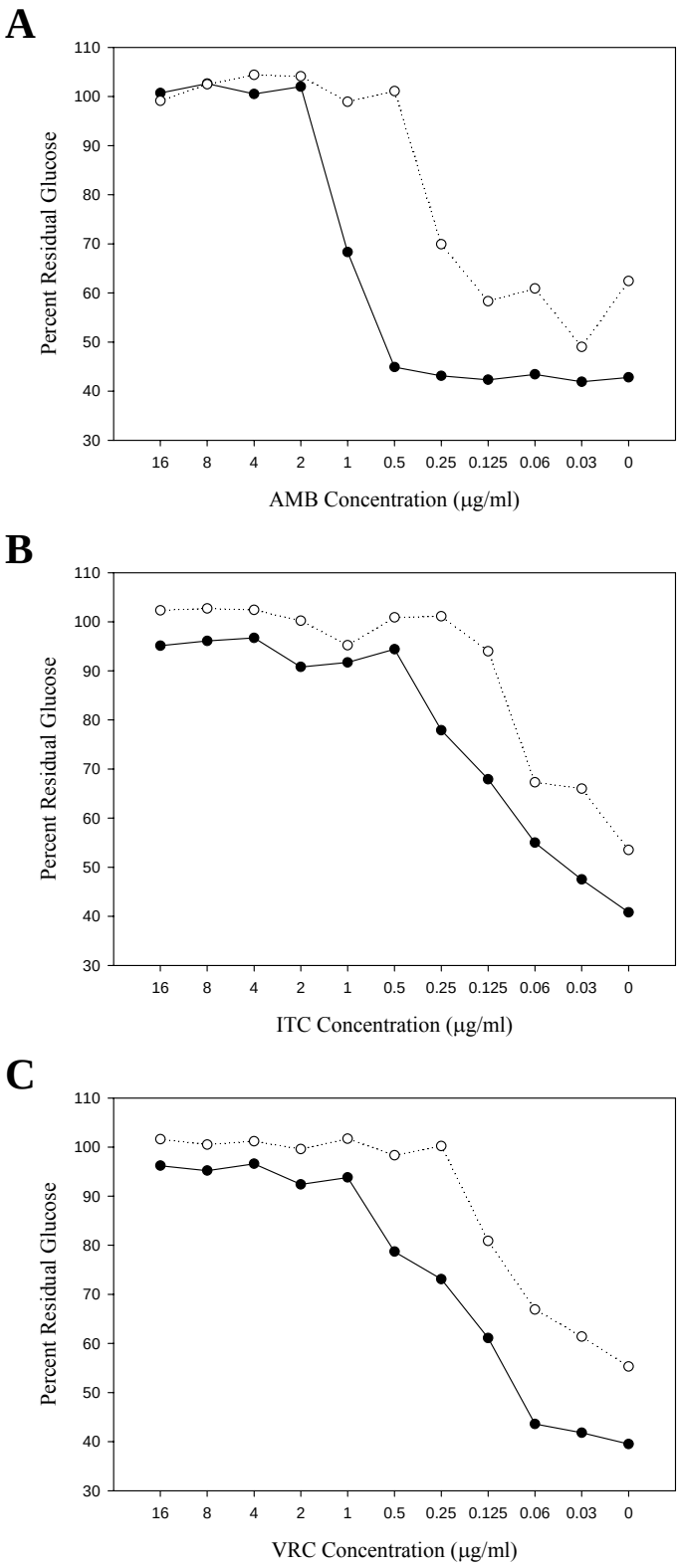


Figure 42. AMB (A), ITC (B), and VRC (C) mRSA susceptibility curves corresponding to 0.11 OD_{530nm} (●) and 1:50 0.11 OD_{530nm} (○) conidial inocula of *A. flavus* AFL3.

Table 15. RSA and NCCLS M38-A MICs of *A. flavus* strains

Strain ID	mRSA MIC[a] (μg/ml)			mRSA MIC[b] (μg/ml)			NCCLS MIC (μg/ml)		
	AMB	ITC	VRC	AMB	ITC	VRC	AMB	ITC	VRC
AF1	0.25	0.125	0.125	0.5	0.25	1	0.5	0.25	0.5
AF2	0.5	0.25	0.25	2	0.25	1	1	0.25	1
AF3	0.5	0.125	0.25	2	0.5	1	1	0.25	0.5

[a] MICs based on analysis of 1:50 0.11 OD_{530nm} mRSA susceptibility curves.
[b] MICs based on analysis of 0.11 OD_{530nm} mRSA susceptibility curves.

interval cutoff of 20%. mRSA MICs obtained with 0.11 OD_{530nm} conidial inocula were equal to or up to eight-fold greater than mRSA MICs obtained with 1:50 0.11 OD_{530nm} conidial inocula. mRSA MICs obtained with 0.11 OD_{530nm} conidial inocula were consistently equal to or within a two-fold dilution of those obtained by NCCLS M38-A testing, while the MICs of 1:50 0.11 OD_{530nm} conidial inocula were up to four-fold less than NCCLS M38-A MICs. However, the only major difference in MIC interpretation between the two mRSA conidial inocula concentrations is that the 0.11 OD_{530nm} conidial inocula of *A. flavus* strains AFL2 and AFL3 each produced AMB MICs of 2 μg/ml, which may indicate AMB resistance, while 1:50 0.11 OD_{530nm} conidial inocula produced AMB MICs of 0.5 μg/ml, indicating AMB sensitivity. Even though *A. flavus* has been documented as developing AMB resistance in vivo (139, 246), I am unable to conclude which MIC interpretation is correct at this time, as the strains tested have no history of clinical outcome. This can be determined in the future by either testing *A. flavus* strains AFL2 or AFL3 in an appropriate animal model to determine responsiveness to AMB in vivo or by performing mRSA AMB testing with the 0.11 OD_{530nm} and 1:50 0.11 OD_{530nm}

conidial inocula of an *A. flavus* clinical control strain, in which the in vivo susceptibility to AMB is known.

CHAPTER 4

MODIFICATION OF MOLD RAPID SUSCEPTIBILITY ASSAY TO DETERMINE SUSCEPTIBILITY OF *ASPERGILLUS FUMIGATUS* HYPHAE TO AMPHOTERICIN B, ITRACONAZOLE, AND VORICONAZOLE

Introduction

In previous work I modified the yeast rapid susceptibility assay (RSA) to determine the susceptibility of *Aspergillus fumigatus* conidia to amphotericin B (AMB) and itraconazole (ITC). The mold RSA (mRSA) is based on the assumption that when fungi are exposed to an inhibitory concentration of antifungal drug, their uptake of nutrients, such as glucose, is suppressed. A minimum inhibitory concentration (MIC) value is determined by comparing the residual glucose levels in the medium following incubation of the fungus with and without drug. This approach provides objective, quantitative MIC values. Importantly, mRSA MIC values obtained at 16 h were equal to or within a single two-fold dilution of MIC values determined by National Committee for Clinical Laboratory Standards (NCCLS) M38-P antifungal testing at 48h.

While most filamentous fungi of medical importance are capable of producing both conidial and hyphal structures, all mold susceptibility tests, including the mRSA, utilize a conidial inoculum (124). Although conidial inocula are easier to standardize compared to hyphal inocula, the use of conidia in standardized susceptibility testing should be questioned for several reasons. Most importantly, branching hyphae are responsible for growth in tissue (35). This means that the use of a conidial inoculum in in vitro antifungal susceptibility testing should only be permitted if the in vitro susceptibility

of conidia is predictive of the susceptibility of hyphae in vivo. Another potential disadvantage of using conidial inocula is that molds isolated in vitro from patients do not necessarily produce conidia. A third important limitation is that conidial production may require an extended incubation period, thus, delaying initiation of susceptibility testing. Results of the limited studies comparing *A. fumigatus* conidial and hyphal MIC values have been controversial (128-130, 132-134, 253), possibly due to the lack of proper control strains and variability of testing parameters such as preparation of hyphal inoculum, hyphal maturity, and inoculum concentration.

To investigate differences in amphotericin B (AMB), itraconazole (ITC), and voriconazole (VRC) MIC values between conidial and hyphal inocula, I modified the mRSA to facilitate susceptibility testing of hyphal inocula (hRSA). As with the mRSA, *A. fumigatus* strains NCPF 7097, 7098, 7100, 7101 were used as hRSA optimizing strains due to their documented in vivo (i.e. hyphal) susceptibilities to AMB or ITC (254, 255). A standardized hRSA hyphal inoculum was generated by inoculating each testing well with a standardized conidia inoculum and incubating at 35-37º C to induce germination. The influence of inoculum size on hRSA MICs was determined by testing hyphal inocula prepared with a range of dilutions of a standardized conidial inocula. Similarities between conidial and hyphal MICs were determined by a direct comparison between mRSA and hRSA MICs, respectively.

Materials and Methods

Reagents

Preparations of RPMI 1640 without glucose (Sigma, St. Louis, MO, cat. no. R-1383) or with 0.4% glucose were buffered with 0.165 M 3-(N-morpholino)-propanesulfonic acid (MOPS) (USB, Cleveland, OH, cat. no. 19256) and adjusted to pH 7.0 with 10 N NaOH.

Fungal Strains

A. fumigatus strains NCPF 7101, 7100, 7098, and 7097 were purchased from the National Collection of Pathogenic Fungi (Mycology Reference Laboratory, Bristol). These strains were isolated from aspergillosis patients with known clinical outcomes. NCPF 7100 and 7098 were isolated from patients with unsuccessful and successful ITC therapeutic outcomes, respectively. NCPF 7097 and 7101 were isolated from patients with unsuccessful and successful AMB therapeutic outcomes, respectively. *A. fumigatus* 204305 was purchased from the American Type Culture Collection (ATCC). Species designations of *A. fumigatus* were confirmed by observations of macroscopic and microscopic characteristics during growth on Czapek-Dox agar (137). All stocks were maintained in vials of sterile water at 4º C.

Antifungal Agents

AMB (Sigma A-9528) and ITC (Research Diagnostics Inc., Flanders, NJ, cat. no. R51211) were purchased in powdered form. VRZ was generously provided by Pfizer,

Inc. (UK). Stocks of 1600 μg/ml were prepared for each drug in dimethyl sulfoxide (DMSO) (Sigma D-2650) and stored at -80°C for up to 6 months.

mRSA and NCCLS M38-A Testing

Antifungal Drug Preparation Serial twofold dilutions of each drug were prepared in DMSO to achieve a drug concentration range of 3 to 1600 μg/ml. Each DMSO dilution was then diluted 1:50 in 0.4% glucose RPMI 1640 to achieve a final 2x drug concentration range of 0.06 to 32 μg/ml. Both drug-free growth and glucose RPMI controls were also prepared, containing the same ratio of DMSO to 0.4% RPMI 1640 as the 2x drug dilutions.

Microwell Plate Preparation Each mRSA and NCCLS M38-A test was performed in duplicate in 96 well round bottom plates (Costar, Corning, NY, cat. no. 3799) with each row, comprised of 12 wells, corresponding to one susceptibility test. All testing was performed three times, in duplicate. Wells 1-10 were inoculated with either 100 μl of the 2 x AMB, ITC, or VRZ dilution ranges (0.06 to 32 μg/ml). Wells 11 and 12, the drug-free growth control and glucose control, respectively, were inoculated with 100 μl of 0.4% glucose-DMSO RPMI 1640. *A. fumigatus* ATCC 204305 was tested in duplicate on every plate as a drug control. Plates were prepared on the day of use and stored at 4º C until needed.

Conidial Inoculum Preparation Isolates were cultured on Sabouraud glucose agar (SAB) (BBL, Cockeysville, MD, cat. no. 211584) slants at 30°C for 3 days. After

flooding with glucose deficient RPMI 1640, the slant surface was gently scraped with a sterile wooden applicator stick. Once the hyphal fragments were allowed to settle for approximately 15 min, the conidial suspension was collected and adjusted with glucose deficient RPMI 1640 to an optical density (OD_{530nm}) of 0.11. The 0.11 OD_{530nm} conidial inoculum of ATCC 204305 was diluted 1:50 with glucose deficient RPMI for NCCLS M38-A testing. Viability of each conidial suspension was determined by plating 0.1ml of the 0.11 OD_{530} conidial inoculum, diluted 1:10,000, on SAB plates and incubating at 37º C.

mRSA assay The microtiter plates were removed from 4º C and allowed to reach room temperature. The mRSA was initiated by inoculating wells 1-11 with 100 µl of 0.11 OD_{530nm} conidial inoculum, well 12 with 100 µl glucose deficient RPMI, and incubation at 35-37º C. At 16h, residual glucose levels were detected by addition of 50 µl of an enzyme color mix comprised of 0.6 M sodium phosphate buffer (pH 6.0), 360 µg/ml of 4-amino antipyrine (4-AAP) (Sigma A4382) , 490 µg/ml of N-ethyl-N-sulfopropyl-m-toluidine (TOPS) (Sigma E8506), 0.68 U/ml horseradish peroxidase (Sigma P8415), and 0.4 U/ml glucose oxidase (ICN Biochemicals, Aurora, OH, cat. no. 195196) to each well. At 20 min, the color intensity was measured at OD_{550nm} with a reference reading of OD_{655nm} in a plate reader (Benchmark Plus, Biorad, Hercules, CA).

mRSA Antifungal Susceptibility Curves and MIC Endpoint Determination The percent residual glucose of each test well was calculated by comparing the OD_{550nm} value of the test well with the OD_{550nm} of the glucose control well based on the following

equation: (OD_{550nm} of test well / OD_{550nm} of glucose control well) X 100. Antifungal susceptibility curves were generated for each RSA by plotting the percent residual glucose values (y-axis) against the appropriate AMB or ITC concentrations (x-axis). The resulting detection interval contained an upper and lower limit represented, respectively, by the highest concentration of antifungal drug and the drug-free growth control. AMB mRSA MIC endpoint determinations were defined as the lowest drug concentration having a percent residual glucose value within the top 10% of the detection interval. The ITC and VRC mRSA MIC endpoints were defined as the lowest drug concentration corresponding to a residual glucose value within the top 20% of the detection interval only if the detection interval was composed of a discernable upper plateau, steep decline, and lower plateau, and the highest concentration of ITC or VRC (16 μg/ml) had a corresponding residual glucose value of ≥ 80%. If these conditions were not met, the ITC or VRC mRSA MIC was interpreted as >16 μg/ml.

NCCLS M38-A Testing and MIC Determination

The NCCLS M38-A assay was initiated by inoculating wells 1-11 with 100 μl of the 1:50 0.11 OD_{530nm} conidial inoculum of ATCC 204305, well 12 with 100 μl of glucose deficient RPMI and incubation at 35-37º C. Although the AMB and ITC dilutions were prepared in 0.4% glucose RPMI and the conidial inocula were prepared in glucose deficient RPMI, the final concentration of glucose was 0.2% at the time of incubation in accordance with M38-A procedures. NCCLS MIC values were determined by visual inspection at 48 h using a mirror plate reader (Cooke Engineering Co., Alexandria, VA) according to NCCLS M38-A guidelines (142) with AMB, ITC, and

VRC MIC endpoint values designated as the lowest drug concentration preventing growth.

hRSA Testing

Antifungal Drug Preparation Serial twofold dilutions of each drug were prepared in DMSO to achieve a concentration range of 1.3 to 656 µg/ml.

Microtiter Plate Preparation 96-well microtiter plates were prepared by adding 100µl of 0.4% glucose RPMI to wells in columns 1-12. Plates were prepared on the day of use and stored at 4º C until needed.

Hyphal Inoculum Preparation A 0.11 OD_{530nm} conidial inoculum was prepared with each strain as described in mRSA testing. Hyphal inocula were prepared by diluting the 0.11 OD_{530nm} conidial inoculum 1:10, 1:50, 1:100, 1:200, and 1:1000 with glucose deficient RPMI. After inoculating 100 µl of appropriate conidial inoculum into wells 1-11 (1-10, test wells; 11, growth control) and 100 µl of glucose deficient RPMI into well 12 (glucose control), the plates were incubated at 35-37º C for 8, 12 or 25 h.

Drug Application Control Immediately before initiating the hRSA, the 12 h hRSA plates were removed from the incubator and the hRSA drug application control (ATCC 204305) set-up in duplicate by adding 100 µl of a 1:50 0.11 OD_{530nm} conidial inoculum to wells 1-11 (1-10, test wells; 11, growth control) and 100 µl of glucose-deficient RPMI to well 12 (glucose control) of the two bottom rows of each hRSA plate.

The drug application control wells were also inoculated with 100 µl of 0.4% glucose RPMI.

hRSA The hRSA was initiated by inoculating 12 h hyphae and ATCC 204305 test wells with 5 µl of the 41x AMB, ITC, or VRZ dilution range (656 down to 1.3 µg/ml), resulting a final concentration range of 0.03 to 16 µg/ml, and growth control and glucose control wells with 5 µl of DMSO. Plates were sealed with tape and shaken at 150 rpm for 15 min. After incubating at 35-37º C for 24 or 48 h, plates were removed and 50 µl of the enzyme color mix (same composition as mRSA) added to each well. At 20 min, color intensity was measured at OD_{550nm} with a reference reading of OD_{655nm} in a plate reader.

hRSA Antifungal Susceptibility Curves and MIC Endpoint Determination The percent residual glucose of each test well was calculated as described in mRSA testing. Antifungal susceptibility curves were generated for each RSA by plotting the percent residual glucose values (y-axis) against the appropriate AMB or ITC concentrations (x-axis). The resulting detection interval contained an upper and lower limit represented, respectively, by the highest concentration of antifungal drug and the drug-free growth control. AMB hRSA MIC endpoint determinations were defined as the lowest drug concentration having a percent residual glucose value within the top 10% of the detection interval. The ITC and VRC hRSA MIC endpoints were defined as the lowest drug concentration corresponding to a residual glucose value within the top 20% of the detection interval only if 1) the detection interval was composed of a discernable upper

plateau, steep decline, and lower plateau, and 2) the highest concentration of ITC or VRC (16 μg/ml) had a corresponding residual glucose value of ≥ 80%. If these conditions were not met, the ITC or VRC mRSA MIC was interpreted as >16 μg/ml.

Statistics Significant differences between mRSA and hRSA AMB, ITC, or VRZ MIC values were determined by the Student t-test. A P-value < 0.05 was considered significant.

Results

Optimal Hyphal Inoculum Conditions

Incubation Period for Hyphal Development The incubation time necessary for development of hyphae from a conidial inoculum was determined by incubating 1:1, 1:10, 1:100, and 1:1000 dilutions of 0.11 OD_{530nm} conidial inocula of *A. fumigatus* NCPF strains 7101, 7100, 7098, and 7097 at 35-37º C and monitoring hyphal development by light microscopy at 8, 12, and 25 h (Figure 43). By 8 h, conidia had germinated and formed immature hyphae (germ tubes) (Fig. 43A). Small aggregates of mature hyphae (hyphae length greater than three times conidial diameter) were observed by 12 h (Figure 43B) with subsequent development of thick hyphal mats on both the fluid surface and microwell walls by 25 h. Similar hyphal development was observed with each strain, regardless of the conidial concentration tested (data not shown). A 12 h incubation period was chosen for subsequent experiments as mature hyphae were observed by this

time period, without the presence of thick hyphal mats on the fluid surface or walls of the well.

Conidial Inoculum Concentration The optimal hRSA conidial inoculum concentration(s) was determined by performing hRSA testing of hyphal inocula prepared

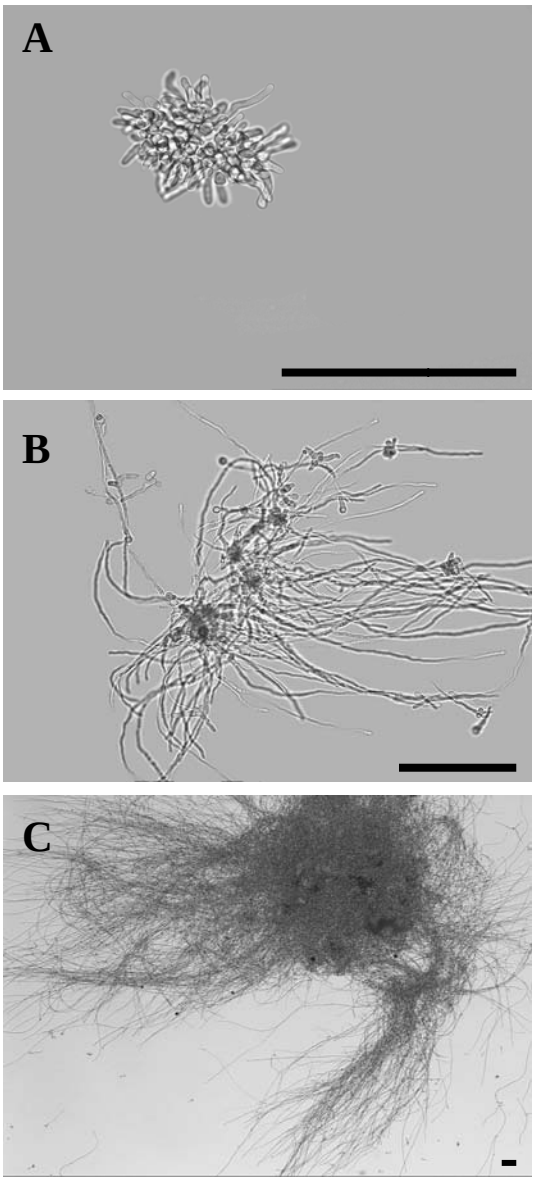


Figure 43. *A. fumigatus* hyphal growth during 25h incubation at 35-37º C. Wet mount slide preps were observed at (A) 8 h, (B) 12 h, (C) and 25 h. Bar represents 150 µm.

from 1:1, 1:10, 1:50, 1:100, 1:200, and 1:1000 dilutions of the 0.11 OD_{530nm} conidial inocula of each *A. fumigatus* strain. At 24 h, hRSA MIC values were not consistently reproducible in these experiments (data not shown), whereas a 48 h incubation resulted in consistent values and was, therefore, chosen as the incubation period for all subsequent hRSA experiments. The AMB, ITC, and VRC MIC values of all strains were influenced by conidial inoculum size, with increasing size corresponding to higher MIC values

Table 16. RSA hyphae AMB, ITC, and VRC MIC value ranges at 48 h[a]

Strain Number	0.11 OD530 C.I. Dilution	Hyphae AMB MIC Range	Hyphae ITC MIC Range	Hyphae VRC MIC Range
NCPF 7098	1:1	>16 µg/ml	>16 µg/ml	>16 µg/ml
	1:10	2	0.25 – 0.5	0.25 – >16
	1:50	0.5 – 1	0.125 – 0.25	0.25 – 0.5
	1:100	0.5 – 1	0.125 – 0.25	0.25
	1:200	0.25	0.125 – 0.25	0.25
	1:1000	0.125 – 0.25	0.125 – 0.25	0.25
NCPF 7100	1:1	4 – >16	>16	>16
	1:10	1 – 2	>16	2
	1:50	0.5	>16	1
	1:100	0.5 – 1	>16	1
	1:200	0.25	>16	1
	1:1000	0.25	>16	1
NCPF 7101	1:1	>16	2 – >16	8 – >16
	1:10	4	0.5	0.5 – 1
	1:50	0.5 – 1	0.25	0.25 – 0.5
	1:100	0.5 – 1	0.125 – 0.25	0.25 – 0.5
	1:200	0.5	0.125 – 0.25	0.25 – 0.5
	1:1000	0.25 – 0.5	0.125 – 0.25	0.25
NCPF 7097	1:1	>16	>16	2 – >16
	1:10	4 – 8	0.5 – 1.0	0.5
	1:50	2	0.25 – 0.5	0.5
	1:100	1	0.125	0.5
	1:200	0.5 – 1	0.125	0.25 – 0.5
	1:1000	0.5 – 1	0.125	0.25

[a] Testing performed three times, in duplicate.

(Table 16). The AMB, ITC, and VRC MIC values corresponding to the 1:1 and 1:10 conidial inocula of strains NCPF 7101 and 7098, in vivo sensitive to AMB (136) and ITC (237), respectively, were significantly higher than those observed with 1:50, 1:100, 1:200, and 1:1000 conidial inocula ($P < 0.05$). ITC hRSA testing of all conidial inocula dilutions of strain NCPF 7100, in vivo resistant to ITC (237), resulted in elevated MIC values (>16 µg/ml) indicating in vitro ITC resistance. AMB and VRC MIC values corresponding to the 1:1 conidial inoculum of strain NCPF 7100 were significantly higher than those observed with the 1:10, 1:50, 1:100, 1:200, and 1:1000 conidial inocula. The ITC and VRC MIC values corresponding to the 1:1 conidial inoculum of strain NCPF 7097, in vivo resistant to AMB (122), were significantly higher than those observed with 1:10, 1:50, 1:100, 1:200, and 1:1000 conidial inocula.

Table 17. Comparison of mRSA and hRSA AMB, ITC, and VRC MICs[a]

Strain Number	Antifungal Drug	mRSA MIC	hRSA MIC	
			1:50[b]	1:200
NCPF 7098	AMB	0.5 µg/ml	1.0 µg/ml	0.25 µg/ml
	ITC	0.5	0.25	0.25
	VRZ	0.25	0.5	0.25
NCPF 7100	AMB	0.5	0.5	0.25
	ITC	>16.0	>16.0	>16.0
	VRZ	0.5	1.0	1.0
NCPF 7101	AMB	0.5	1.0	0.5
	ITC	0.5	0.25	0.25
	VRZ	0.25	0.5	0.5
NCPF 7097	AMB	0.5	2.0[c]	1.0
	ITC	0.5	0.5	0.25
	VRZ	0.25	0.5	0.5

[a] Geometric mean. Testing performed three times, in duplicate.
[b] 0.11 OD_{530} conidial inoculum dilution used in hyphal inoculum preparation.
[c] hRSA MIC significantly higher than mRSA MIC.

Comparison of Conidial and Hyphal MICs The mRSA and hRSA (1:50 and 1:200 dilutions of 0.11 OD_{530nm} conidial inocula) AMB, ITC, and VRC MIC values of each strain are shown in Table 17. With one exception, hRSA AMB, ITC, and VRC MIC values were within a single two-fold dilution of AMB, ITC, and VRC MIC values obtained with the mRSA. The hRSA AMB MIC value of NCPF 7097, obtained with a 1:50 0.11 OD_{530nm} conidial inoculum, was four-fold higher than the AMB MIC value obtained by the mRSA.

Discussion

The goal of this study was to compare the AMB, ITC, and VRC susceptibilities of *A. fumigatus* conidia and hyphae. This was accomplished by modifying the mRSA, which utilizes conidial inocula, to facilitate susceptibility testing of hyphal inocula. mRSA factors requiring modification included inoculum preparation, incubation time, and MIC endpoint determination.

There are no guidelines for preparing a standardized *Aspergillus* hyphal inoculum for broth microdilution susceptibility testing. Pujol (129), Manavathu (128), and Espinel –Ingroff (130) et al. have prepared hyphal inocula by inoculating a single volume of growth medium broth (Czapek, peptone-yeast extract-glucose, or RPMI, respectively) with a standardized conidial suspension and incubating, with or without shaking, until hyphae developed. The broth was next vortexed, to fragment the hyphae, and standardized by CFU/ml or optical density. A limitation of this method is that the hyphae are randomly fragmented by vortexing, potentially resulting in batch-to-batch differences

in the amount or length of hyphae in the hyphal inocula and physical damage to the hyphae that may affect the viability or drug susceptibility of hyphae (256). In contrast, Lass-Florl (132, 134, 253) et al., prepared hyphal inocula by inoculating 96-well plates with a standardized conidial inoculum, incubating to allow formation of hyphae, then washing and refilling the wells with fresh growth medium containing antifungal drug. While this method uses non-fragmented hyphae, the washing step may be problematic. During hyphal formation, hyphae may attach to the bottom and sides of the well as well as initiate formation of a hyphal mat on the fluid surface. As Lass-Florl allowed 16-22 h for hyphal formation, a sufficient time for early development of a hyphal mat on the fluid surface, well washing may have resulted in removal of the hyphal mat and other hyphae loosely bound to well walls. As both methods are problematic, I chose a third method of hyphal inoculum preparation.

Like Lass-Florl, 100 µl of conidial inoculum was inoculated into 96-well plates containing 100 µl of RPMI and incubated at 35-37º C to allow formation of hyphae. I chose an incubation time of 12 h for hyphal formation (Fig. 43) as hyphae were still young germ tubes at 8 h (Fig. 43A) and because I was concerned that hyphal mat growth on the fluid surface was too extensive at 25 h (Fig. 43C), possibly interfering with drug addition. To retard evaporation during the initial 12 h incubation, I sealed the plate edges with tape, resulting in evaporation of ≤10 µl in any given well (data not shown). Instead of washing the wells immediately after the 12 h incubation, a small volume (5 µl) of drug was added to the existing media in each well, leaving the hyphae undisturbed. By visually examining wells containing 16 µg/ml AMB (an antifungal drug with yellow

pigmentation), I determined that antifungal drug could be evenly dispersed in each well by shaking plates at 150 rpm for 15 min. The plates were then incubated for an additional 48 h, with residual glucose levels being determined by the same methodology used in mRSA testing. To confirm that differences between mRSA and hRSA MICs were not due to differences in each assay's method of drug addition, I included a drug addition control in hRSA testing by inoculating the 1:50 0.11 OD_{530nm} conidial inoculum of ATCC 204305 to the bottom two rows of each hRSA plate prior to drug addition. The 48 h AMB, ITC, and VRC MICs of this drug addition control were equal to or within one two-fold dilution of those obtained with 16 h mRSA testing and 48 h NCCLS M38-A testing (data not shown). As I consistently observed a non-significant ±1 two-fold dilution MIC difference in mRSA and NCCLS M38-A testing, I concluded that there were no significant differences between MICs obtained by the mRSA and hRSA methods of drug addition.

To ensure the accuracy of our data interpretation, I optimized hRSA testing factors using clinical *A. fumigatus* strains with documented clinical and therapeutic patient histories. The optimal range of conidial inoculum concentrations for each of these clinical strains corresponded to in vitro MICs that were non-dose dependent (not affected by conidial inoculum concentration), differing in value by no more than two, two-fold dilutions, and reflective of the documented in vivo AMB or ITC susceptibilities of the strain. *A. fumigatus* strains NCPF 7101 and 7098 were obtained from aspergillosis patients who responded to AMB and ITC therapy, respectively (136, 237). The AMB MICs corresponding to the 1:1 and 1:10 conidial inocula of strain NCPF 7101 and ITC

MICs corresponding to the 1:10 conidial inocula of strain NCPF 7098 did not indicate in vitro sensitivity to AMB or ITC, respectively, and increased in a concentration dependent manner (Table 16). The 1:50 to 1:1000 dilutions of NCPF 7101 0.11 OD_{530nm} conidial inocula and 1:10 to 1:1000 dilutions of NCPF 7098 0.11 OD_{530nm} conidial inocula were considered optimal as the corresponding MICs indicated in vitro sensitivity to AMB and ITC, respectively, and were not dose-dependent (± 2 dilutions). NCPF 7097 was isolated from a patient that initially responded to AMB therapy but later relapsed (136), while NCPF 7100 was obtained from a patient that did not respond to primary ITC therapy (237). Although the 1:1, 1:10, and 1:50 0.11 OD_{530nm} conidial inocula of NCPF 7097 corresponded to MICs that are equal to or greater than Lass Florl's presumptive AMB breakpoint value of ≥ 2 μg/ml (123), they are dose dependent and therefore artifactual. As AMB resistance has not been consistently demonstrated in vitro, it was not surprising that the majority of the optimal conidial concentrations (1:50, 1:100, 1:200, 1:1000 0.11 OD_{530nm}) corresponded to AMB MICs that indicated AMB sensitivity. The ITC MICs of NCPF 7100 consistently indicated in vitro ITC resistance, regardless of conidial inoculum concentration, indicating that all conidial inocula concentrations could be considered optimal. The final range of conidial inoculum concentrations common to the optimal conidial inoculum concentration range of each strain included the 0.11 OD_{530nm} dilutions 1:50, 1:100, 1:200, and 1:1000.

As VRC was only recently approved for use, I was unable to obtain in vivo VRC-sensitive or -resistant strains to optimize VRC hRSA conditions. This may not be necessary, however, as previous testing has shown that optimal mRSA VRC conditions

are identical to optimal mRSA ITC conditions, with verification of these conditions as optimal by demonstrating that the 16 h mRSA VRZ MICs were equal to or within one two-fold dilution of those obtained by NCCLS M38-A testing at 48 h (data not shown). By analyzing the VRC MICs obtained with a range of conidial inocula concentrations (Table 16), I was able to determine that VRC MICs were dose-dependent, with 1:10 and/or 1:1 0.11 OD_{530nm} conidial inocula producing erroneously high MICs and 1:50-1:1000 0.11 OD_{530nm} conidial inocula producing MICs indicating VRC sensitivity and differing by no more than three two-fold dilutions. The latter dilutions were consistent with those conidial inocula concentrations considered optimal for AMB and ITC testing.

To determine whether hyphae sensitivity to AMB, ITC, and VRC was significantly different to that of conidia, I compared mRSA AMB, ITC, and VRC MICs with hRSA MICs (1:50 and 1:200 conidial inoculum dilutions) (Table 17). With one exception, I found that there were no significant differences between mRSA and hRSA MICs. The exception was the AMB MIC corresponding to the 1:50 0.11 OD_{530nm} conidial inoculum of NCPF 7097, which was significantly higher (p-value <0.05) than the mRSA AMB MIC. Either the hRSA 1:50 0.11 OD_{530nm} conidial inoculum is too concentrated and corresponds to an artificially high MIC or AMB resistance can be detected more readily with hyphae than conidia. As the hRSA 1:200 0.11 OD_{530nm} AMB MIC was not significantly greater than the mRSA AMB MIC, it is more probable that the former is correct, but additional hRSA and mRSA testing of other clinical AMB-resistant strains of *A. fumigatus* should be tested to confirm this.

These data show that hyphae and conidia have similar sensitivities to AMB, ITC, and VRC, suggesting that either fungal form can be used to preparing inocula for antifungal susceptibility testing. As hRSA testing requires 44 more hours than mRSA testing, I recommend the continued use of the conidial inocula in the mRSA. Because a conidial inoculum was necessary for performing the hRSA, a future goal is to develop another hRSA protocol that utilizes a standardized hyphal inoculum prepared with hyphae instead of conidia. This version of the hRSA will be suitable for testing those clinical isolates which can not be tested in the mRSA, as they do not sporulate in vitro.

CHAPTER 5

SIDE-BY-SIDE COMPARISON OF AMPHOTERICIN B, ITRACONAZOLE, AND VORICONAZOLE MINIMUM INHIBITORY CONCENTRATION VALUES OBTAINED BY MOLD RSA AND ETEST ANTIFUNGAL SUSCEPTIBILITY TESTING

Introduction

The National Committee for Clinical Laboratory Standards (NCCLS) M38-A assay is the standardized assay most frequently used in antifungal susceptibility testing of filamentous fungi. Unfortunately, the NCCLS M38-A assay is burdensome and time-consuming, prompting investigators to find alternative methods. The Elipsometer Test (Etest) is a patented method for the quantitative determination of antimicrobial minimum inhibitory concentration (MIC) values. The mold Etest is similar to other agar-diffusion based susceptibility tests, except that the disk is replaced by a plastic strip which is impregnated with a gradient of antifungal drug (149).

Although numerous studies have been conducted to determine the percent agreement between the NCCLS M27-A2 and Etest MICs of *Candida* and *Cryptococcus* spp. (152, 154, 155, 157-160, 257-261), those determining the percent agreement between NCCLS M38-A and Etest MICs of *Aspergillus* spp. have been more limited (149-151, 156, 262, 263). Overall, the results of these *Aspergillus* studies have demonstrated that amphotericin B (AMB), itraconazole (ITC), voriconazole (VRC), and posaconazole (PCZ) NCCLS M38-A MICs have moderate to excellent agreement (60-100%) with Etest MICs, with results depending on Etest factors such as agar medium and incubation time. As the 16 h mold Rapid Susceptibility Assay (mRSA) AMB, ITC, and

VRC MICs have been shown to have an excellent agreement (100%) with NCCLS M38-P and M38-A MICs (Chapters 2 and 4, respectively), I determined whether mRSA AMB, ITC, and VRC MICs would also demonstrate a high percentage agreement with Etest MICs.

Materials and Methods

Reagents

For mRSA testing, preparations of RPMI 1640 with or without 0.4% glucose were buffered with 0.165 M 3-(*N*-morpholino)-propanesulfonic acid and adjusted to pH 7.0 with 10 N NaOH. 90 or 150 mm petri plates containing 2.0% glucose RPMI 1640 agar (R63165; Angus Biochemicals, Niagara Falls, NY) were used for Etest testing.

Fungal Strains

A. fumigatus testing strains included the in vivo AMB-sensitive and –resistant strains 7101 and 7097, respectively, and the in vivo ITC-sensitive and –resistant strains 7098 and 7100, respectively (136, 237). *C. parapsilosis* ATCC 22019 was used as an Etest drug control. All *A. fumigatus* stocks were maintained in vials of sterile water at 4º C. The *C. parapsilosis* stock was maintained in a vial of sterile water at room temperature.

Antifungal Drugs and Preparation

AMB, ITC and VRC Etest strips were purchased from AB Biodisk NA, Inc. and stored at -20º C until use. AMB and ITC were purchased from Sigma and Research

Diagnostics Inc., respectively, while VRC was generously provided by Pfizer (UK). Stocks of 1600 µg/ml were prepared in dimethyl sulfoxide (DMSO) and stored at -80º C for up to six months. For mRSA testing, serial two-fold dilutions were prepared and loaded into the wells of 96-well microwell plates as described in the "Antifungal Agents and Dilutions" and "Microwell Plate Preparation" sections of Chapter 2. mRSA drug plates were prepared on the day of use and stored at 4º C until use.

Inoculum Preparation

A. fumigatus isolates were cultured on Sabouraud agar slants at 30º C for 3 days. *C. parapsilosis* ATCC 22019 was inoculated onto a GYEP plate and passaged twice at 24 h intervals. For mRSA and Etest testing, slants were flooded with glucose deficient RPMI or sterile saline, respectively, and the slant surface scraped with a sterile applicator stick. Once the hyphal fragments had settled (15 min), the upper conidial inoculum was collected and adjusted with additional glucose deficient or saline to an optimal density (OD_{530nm}) of 0.11. NCCLS M38-A inocula were prepared by diluting the 0.11 OD_{530nm} conidial inocula 1:50 with additional glucose-deficient RPMI. The *C. parapsilosis* inoculum was prepared by transferring yeast cells from 1 to 2 colonies on the 24 h GYEP plate to glucose deficient RPMI and adjusting the optical density (OD_{530nm}) to achieve a density of 0.11. Etest and mRSA inocula were kept at 4º C until use.

Etest Assay and MIC Determination

Each 90 or 150 mm 2.0% glucose RPMI agar plate was inoculated by dipping a sterile swab into the 0.11 OD_{530nm} inoculum suspension, rolling the swab against the side

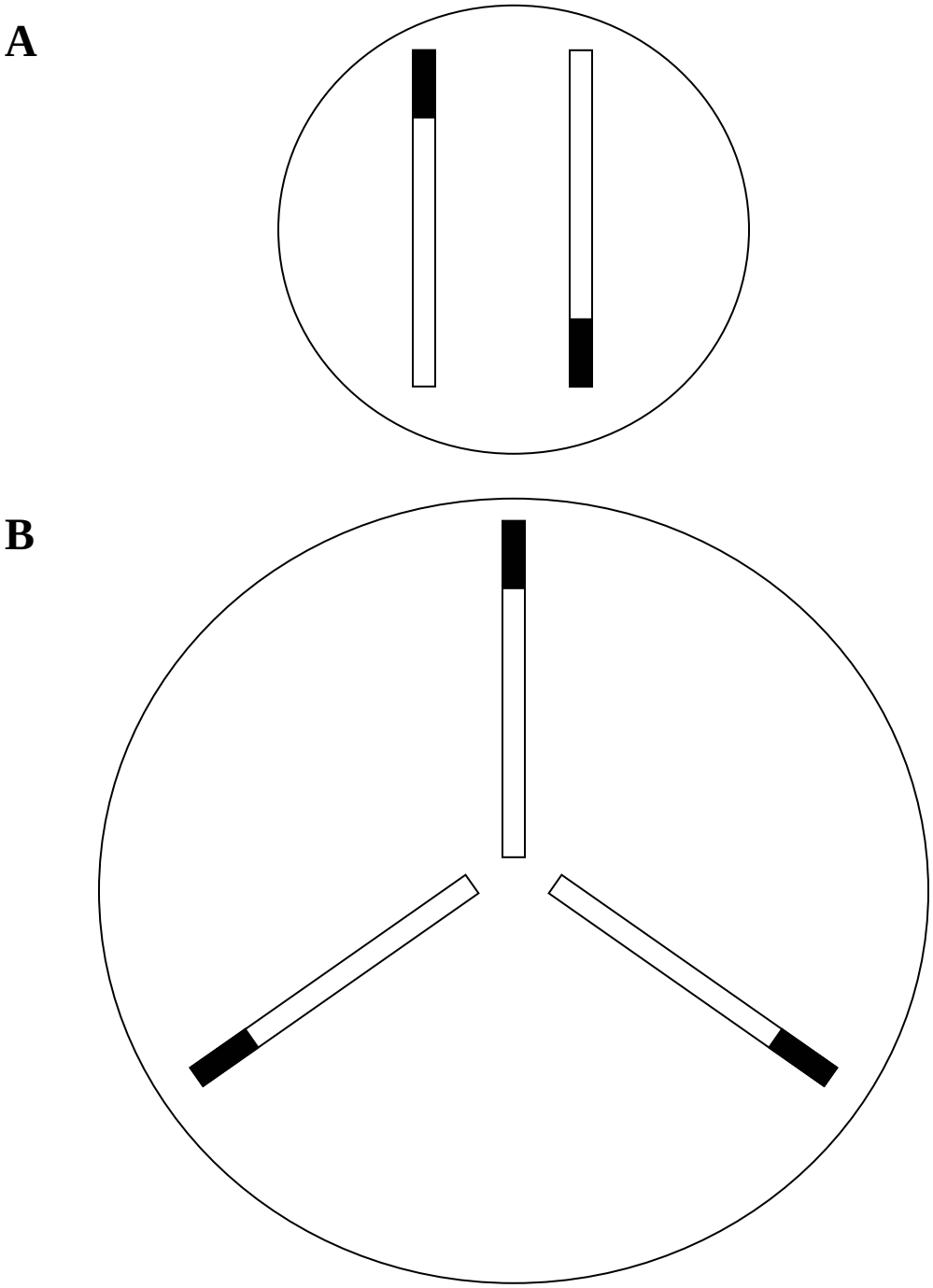


Figure 44. Correct placement of Etest strips on surface of 90 mm (A) and 150 mm (B) 2.0% glucose RPMI agar plates. Each Etest strip is place on the agar surface with the MIC scale (white portion of strip) facing upwards and the highest drug concentration oriented toward the plate rim.

of the tube to remove excess moisture, and swabbing the entire agar surface in three directions. After the plate surface dried for 10-15 min, two (90 mm plate) or three (150 mm plate) Etest strips were placed on the agar surface (Fig. 44). Plate edges were sealed with parafilm and incubated at 35-37º C for 24-48 h. MICs were read at 24 and 48 h. AMB, ITC, and VRC MICs were designated as the lowest drug concentration at which the border of the elliptical inhibition zone intercepted the scale on the Etest strip (Fig. 45). All *C. parapsilosis* AMB, ITC, and VRC MICs were within the MIC ranges recommended by AB Biodisk (data not shown).

mRSA Assay and MIC Determination

The 16 h mRSA was performed, and AMB and ITC MICs determined, as described in the Materials and Methods "RSA" section of Chapter 2. VRC MICs were determined as described in the Materials and Methods "VRC MIC Determination" section of Chapter 3.

Percent Agreement Between Etest and mRSA MICs

Etest MICs read at 24 or 48 h were compared to mRSA MICs obtained in 16 h. Since Etest strips are composed of a drug gradient, the MICs in between two-fold mRSA dilutions were rounded up to the next two-fold mRSA drug concentration for comparison. Discrepancies between Etest and mRSA MICs of no more than two two-fold dilutions (ex. 0.5, 1.0, 2.0 µg/ml) were used to calculate the percent agreement (151, 242, 252, 264).

Results and Discussion

As previous studies have recommended a 24 versus 48 h incubation period for determination of Etest AMB MICs {20, 500, 443), I compared the 24 and 48 h inhibition growth inhibition ellipse patterns and AMB MICs of AMB-sensitive and –resistant strains NCPF 7101 and 7097, respectively, to determine which incubation period was optimal (Fig. 46). By 48 h, the inhibition ellipse size had greatly reduced both horizontally and vertically (Fig. 46B, 47B), resulting in an erroneously high AMB MIC for the AMB-sensitive *A. fumigatus* strain NCPF 7101 (Fig. 47B). Subsequent AMB Etest testing was therefore performed in 24 h while ITC and VRC testing was

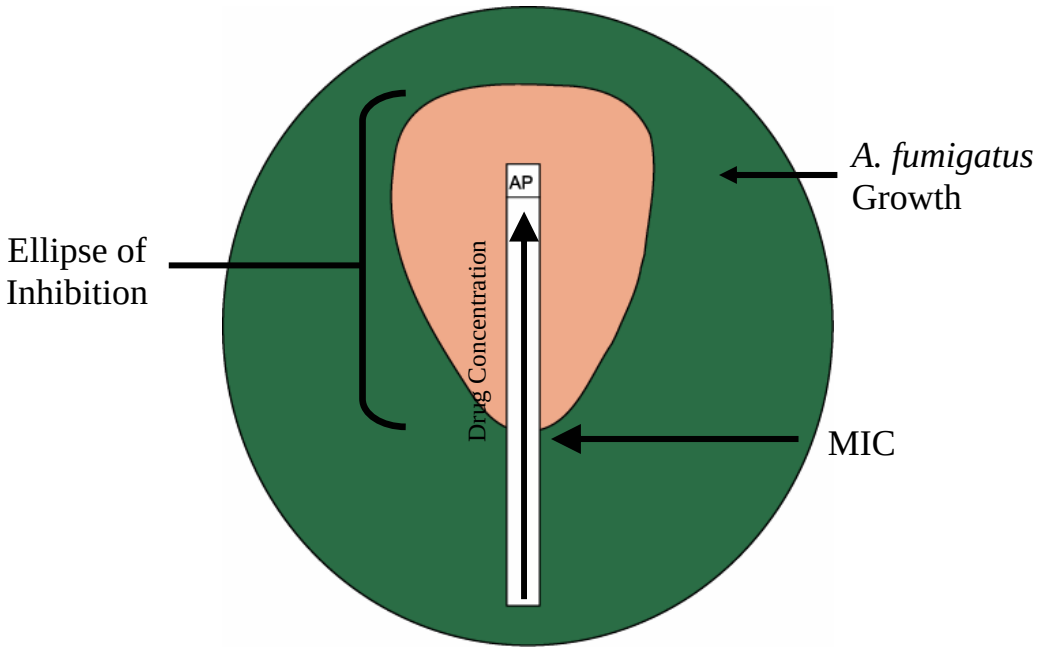


Figure 45. Designation of Etest MIC. Concentrations of drug inhibiting fungus growth generate a visible ellipse of inhibition. The MIC is the lowest drug concentration at which the ellipse border intercepts the scale on the Etest strip.

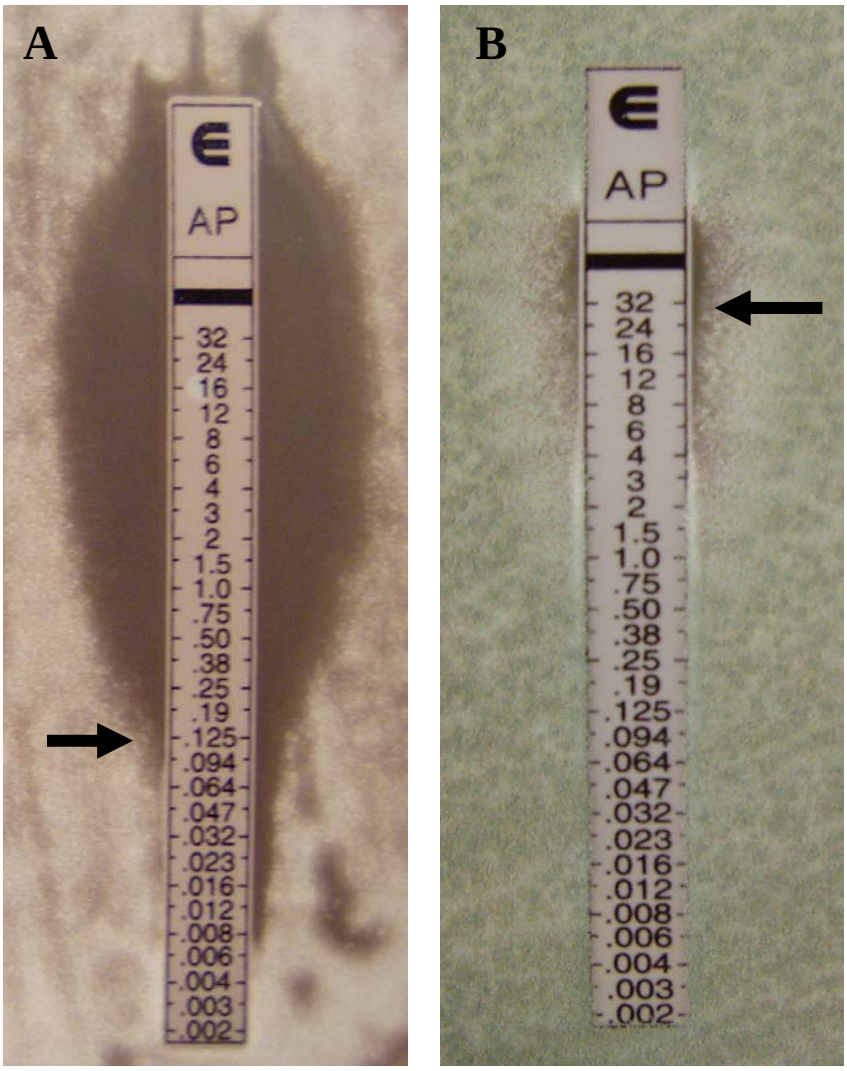


Figure 46. Differences in 24 h (A) and 48 h (B) Etest AMB MICs of AMB-resistant *A. fumigatus* strain NCPF 7097. AMB MIC indicated by arrow.

performed in 48 h.

All 24 and 48 h inhibition ellipses were clear (i.e. no trailing growth), with the comparative widths of 48 h ellipses between drugs being VRC > ITC > AMB (Fig. 48). The absence of trailing growth was not surprising as AMB, ITC, and VRC are fungicidal

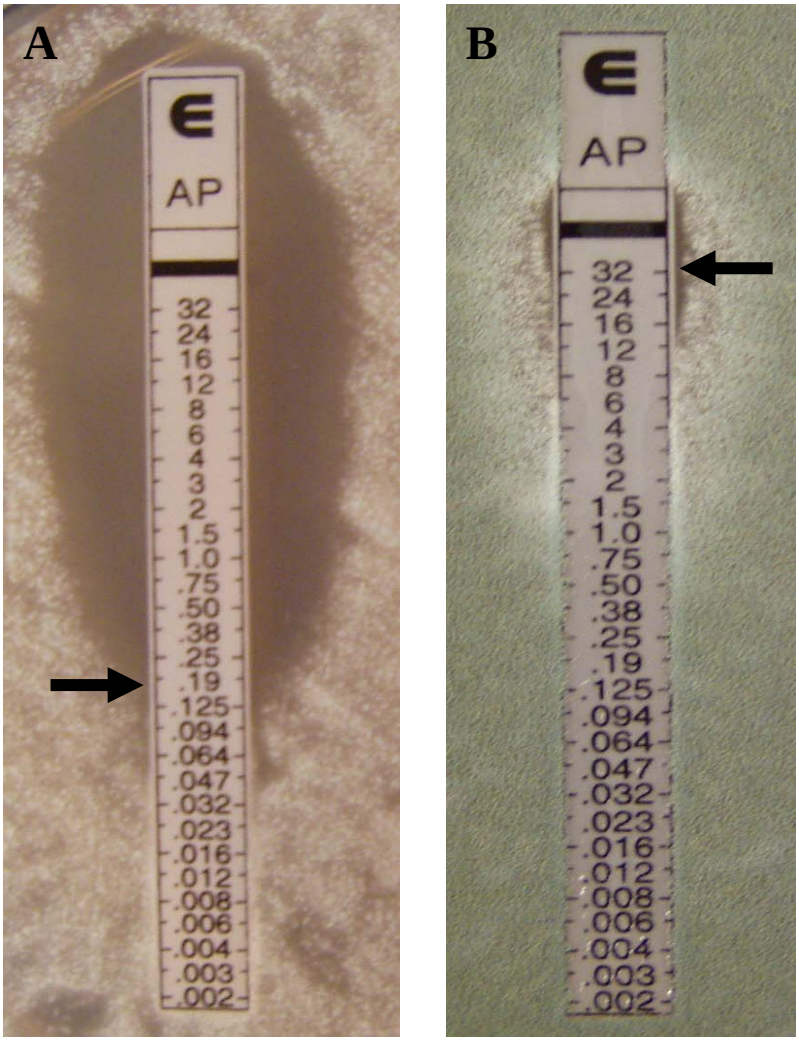


Figure 47. Differences in the 24 h (A) and 48 h (B) Etest AMB MICs of AMB-sensitive *A. fumigatus* strain NCPF 7101. AMB MIC indicated by arrow.

against *Aspergillus fumigatus*. Also, the relative ellipse sizes between drugs can be explained by the drugs' molecular weights, with VRC and AMB having the smallest and largest molecular weights, respectively.

Etest and mRSA AMB, ITC, and VRC MICs are shown in Table 18. The Etest

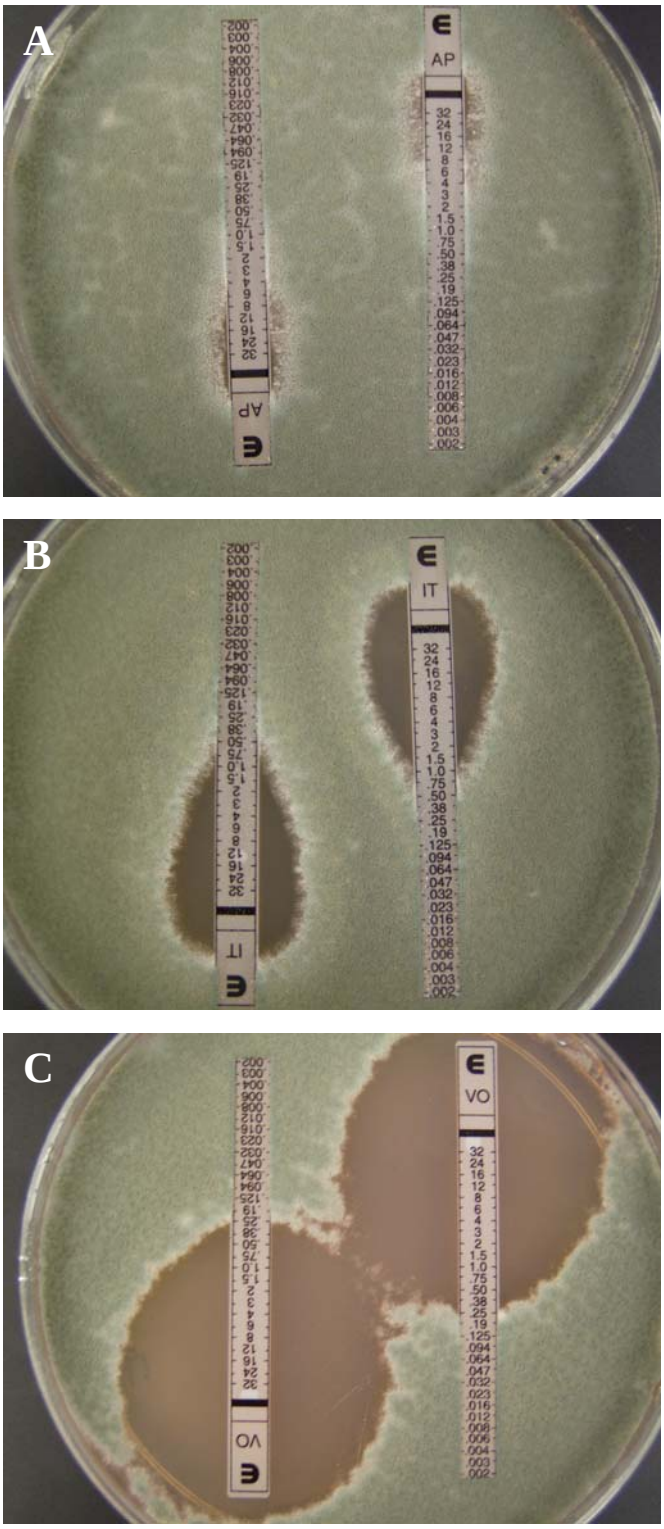


Figure 48. Comparison of 48 h AMB (A), ITC (B), and VRC (C) inhibition ellipse sizes.

Table 18. *A. fumigatus* Etest and mRSA AMB, ITC, and VRC MICs

A. fumigatus NCPF strain	Antifungal Drug	Incubation Time[a]	MIC (µg/ml)[b]	
			Etest	mRSA
7101	AMB	24 h	0.19	0.5
	ITC	48 h	1.5	0.5
	VRC	48 h	0.25	0.25
7100	AMB	24 h	0.25	0.5
	ITC	48 h	>32	>16
	VRC	48 h	3	0.5
7098	AMB	24 h	0.064	0.5
	ITC	48 h	1	0.5
	VRC	48 h	0.25	0.25
7097	AMB	24 h	0.125	0.5
	ITC	48 h	0.75	0.5
	VRC	48 h	0.25	0.25

[a] Indicates Etest incubation time. All mRSA testing was performed in 16 h.
[b] Both Etest and mRSA testing performed three times, each time in duplicate. The MICs represent geometric means.

did not detect AMB resistance, as the 24 h AMB MIC of the AMB-resistant *A. fumigatus* strain 7097 was 0.125 µg/ml, indicating AMB sensitivity. Previous AMB Etest testing of NCPF 7097, performed by Denning et al., also resulted in a lack of AMB resistance detection (136). In contrast, the Etest did detect ITC resistance, as the 48 h AMB MIC of the ITC-resistant *A. fumigatus* strain 7100 was >32 µg/ml. AMB and ITC Etest MICs were in 100% agreement with mRSA AMB and ITC MICs while VRC Etest MICs were in 75% agreement with mRSA VRC MICs. The only discrepancy between Etest and mRSA VRC MICs was observed with the ITC-resistant *A. fumigatus* strain 7100, whose Etest MIC (3 µg/ml) was 2.5x greater than its corresponding mRSA MIC (0.5 µg/ml). It

is possible that the Etest VRC MIC is erroneously high. However, this seems improbable as none of the ITC-sensitive *A. fumigatus* strains exhibited elevated Etest VRC MICs. Instead, it seems more probable that the Etest is a more sensitive assay than the NCCLS M38-A or mRSA assays in detecting VRC-ITC cross-sensitivity. VRC Etest testing of additional ITC-resistant *A. fumigatus* strains will be necessary to resolve this issue.

In summary, *A. fumigatus* mRSA MICs demonstrated excellent agreement (92%) with Etest MICs. In contrast to yeast testing (157, 158), mold Etest testing was unable to detect AMB resistance, although the Etest did generated ITC MICs that could be used as predictors of in vivo outcome. Despite the fact that the Etest is less cumbersome than the mRSA, I still propose that the mRSA is a superior assay, as it generates objective MICs in 16 h versus the subjective MICs generated by the Etest in 24 or 48 h.

CHAPTER 6

VALIDATION AND EXPANSION OF YEAST RSA

Introduction

The gold-standard method for yeast antifungal susceptibility testing is the National Committee for Clinical Laboratory Standards (NCCLS) M27-A2 assay, which provides amphotericin B (AMB), flucytosine (5FC), fluconazole (FLC), ketoconazole (KET), itraconazole (ITC), posaconazole (POS), ravuconazole (RAV), and voriconazole (VRC) minimum inhibitory concentration (MIC) values for *Candida* spp. (including *C. glabrata*) and *Cryptococcus neoformans* within 48 and 72 h, respectively (135). However, the NCCLS M27-A2 assay has major weaknesses, including subjective MIC determination and extended incubation periods. The yeast Rapid Susceptibility Assay (RSA) was developed in our laboratory to address these weaknesses, by providing objective AMB and FLC MICs in 6 and 19 h, respectively, for *C. albicans*, *C. tropicalis*, *C. parapsilosis*, and *C. krusei* isolates (145). Unfortunately, RSA testing conditions were not optimized with clinical control strains, which compromises the validity of RSA MICs as predictors of clinical outcome. Another current disadvantage of the RSA is that is does not include testing of *C. glabrata*, a species which has rapidly increased in clinical relevance in the past decade.

Clinical control strains are those isolated from patients with documented clinical outcomes. In theory, strains obtained from patients with successful clinical outcomes correspond to low MICs, while strains obtained from patients with clinical failure

correspond to high MICs. Clinical control strains are therefore useful in optimizing antifungal susceptibility testing conditions because they ensure that that the in vitro MICs are predictive of clinical outcome. Instead of using clinical control strains, RSA optimized testing conditions using NCCLS M27-A2 recommended quality control (QC) or reference strains: ATCC 24433 and ATCC 90028 (*C. albicans*); ATCC 750 (*C. tropicalis*); ATCC 6258 (*C. krusei*); and ATCC 22019 and 90018 (*C. parapsilosis*) (135). Although the ATCC database identifies these strains as clinical isolates, it does not provide therapeutic or clinical outcome histories of the patients from which the strains were isolated. These strains were chosen because the NCCLS M27-A MICs of these strains were highly reproducible and available in the literature (265), which facilitated a direct comparison between RSA MICs and published NCCLS M27-A MICs. Although the yeast RSA can, in its current form, detect innate FLC resistance, as demonstrated by the elevated RSA FLC MIC (32-64 μg/ml) of *C. krusei* ATCC 6258 (a species innately resistant to FLC in vitro and in vivo), it does not guarantee that testing conditions are optimized to detect acquired FLC resistance. By determining the yeast RSA FLC MICs of FLC-resistant and –sensitive C. *albicans* clinical control strains, I will be able to demonstrate whether the RSA, in its current form, has the capability of predicting FLC resistance in vitro, and more importantly, in vivo.

The ability to determine *C. glabrata* FLC MICs by RSA testing is essential due to the increasing frequency of mucosal and systemic candidiasis caused by *C. glabrata* in the past decade (266). Due to the widespread and increased use of immunosuppressive therapy and broad-spectrum antifungal therapy, *C. glabrata* is now the second most

frequent etiologic agent of candidiasis (11, 19, 20, 32, 216) in the Unites States. To ensure that *C. glabrata* RSA FLC MICs are predictive of clinical outcome, I optimized RSA testing conditions using *C. glabrata* clinical control strains that were isolated from oropharyngeal candidiasis patients receiving head and neck cancer therapy at the Cancer Therapy and Research Center in San Antonio (personal communication with Dr. Spencer Redding). Optimized testing conditions were considered those producing MICs that reflected the clinical outcome history of each strain.

Materials and Methods

Reagents

Preparations of RPMI 1640 without glucose or with 0.44% glucose were buffered with 0.165 M 3-(N-morpholino)-propanesulfonic acid (MOPS) and adjusted to pH 7.0 with 10 N NaOH.

Fungal Strains

Six *C. albicans* and seven *C. glabrata* clinical strains were used as clinical controls in RSA and NCCLS M27-A2 testing. Dr. Ted White provided *C. albicans* FH1 and FH5, FLC-susceptible and -resistant isolates, respectively, which were derived from the same *C. albicans* strain causing invasive candidiasis in a bone marrow transplant patient (267). Dr. Spencer Redding provided *C. albicans* strains 1 and 17 and *C. glabrata* strains 6318, 6838, 6844, 6856, 6955, 6999, 7015, and 7048. *C. albicans* strains 1 and 17, FLC-susceptible and resistant, respectively, were derived from the same *C. albicans* strain causing oropharyngeal candidiasis in an AIDS patient (268). Originally

isolated by Dr. David Stevens, Dr. Ted White also provided FLC-resistant *C. albicans* strains 95-68 and 95-142, which are random clinical isolates (269). All FLC-resistant *C. albicans* strains were previously confirmed as such by identification of resistance mechanisms, including FLC efflux pumps and overexpression and/or DNA point mutations of FLC target (269, 270). *C. glabrata* strains 6838, 6844, 6856, and 6955 were sequentially obtained during the FLC treatment of a throat cancer patient with oropharyngeal candidiasis (271). *C. glabrata* strains 6999, 7015, and 7048 were also obtained sequentially during the FLC treatment of a throat cancer patient with oropharyngeal candidiasis. *C. glabrata* strain 6318 was a random clinical isolate (personal communication with Dr. Spencer Redding). *C. krusei* ATCC 6258 is a NCCLS M27-A2 reference strain and was used as a FLC drug control for RSA and NCCLS M27-A2 testing. Strains were confirmed as *C. albicans*, *C. glabrata*, or *C. krusei* by ChromAgar and/or API testing.

Fluconazole Stock and Preparation

FLC was purchased in a powdered form. A stock of 1600 μg/ml was prepared in sterile water and stored at -80º C for up to six months. For RSA testing, a serial two-fold dilution of FLC was prepared in sterile water to achieve a concentration range of 2.5 to 1280 μg/ml. For NCCLS M27-A2 testing, each water dilution was diluted 1:10 in 0.44% RPMI 1640 to achieve a final 2x drug concentration range of 0.25 to 128 μg/ml in 0.4% glucose RPMI. For RSA testing, each water dilution was diluted 1:10 in 0.22% RPMI 1640 to achieve a final 2x drug concentration range of 0.25 to 128 μg/ml in 0.2% glucose RPMI.

Microwell Plate Preparation

Each RSA and NCCLS M27-A2 test was performed in duplicate in 96 well round bottom plates with each row, comprised of 12 wells, corresponding to one susceptibility test. Wells 1-10 were inoculated with either 100 µl of the 2x FLC dilution range (0.25 to 128 µg/ml). Wells 11 and 12, the drug-free growth control and glucose control, respectively, were inoculated with 100 µl of 0.4% glucose RPMI 1640. Plates were prepared on the day of use and stored at 4º C until needed.

Inoculum Preparation

Yeasts were inoculated onto GYEP plates from room temperature water stocks and passaged twice at 24 or 48 h intervals. Suspensions were prepared from 24 h cultures. Cells from colonies were suspended in glucose deficient RPMI and the optical density (OD_{530nm}) adjusted to achieve a density of 0.11. Yeast viability was determined by plating 0.1ml of a 1:10,000 dilution of the 0.11 OD_{530nm} yeast inoculum of each strain on GYEP plates, incubating at 35-37° C for 24 to 48 h, and calculating the CFU/ml.

The published optimal inoculum dilution range for *C. albicans* RSA FLC testing is 1:512 to 1:8192 0.11 OD_{530nm}. I chose to use the same inoculum concentration of 1:1000 0.11 OD_{530nm} used in NCCLS M27-A2 testing, which was prepared by diluting the 0.11 OD_{530nm} yeast inoculum 1:50, then 1:20 with glucose deficient RPMI to achieve a final concentration of 1×10^3 to 5×10^3 CFU/ml. *C. glabrata* RSA inocula were prepared by diluting 0.11 OD_{530nm} yeast inocula 1:2, 1:8, 1:128, 1:512, 1:1000 or 1:2048 with glucose deficient RPMI. *C. albicans*, *C. glabrata*, and *C. krusei* NCCLS M27-A2 inocula were prepared by diluting the 0.11 OD_{530nm} conidial inoculum 1:50, then 1:20

with glucose deficient RPMI. Both RSA and NCCLS inocula were stored at 4° C until use.

RSA Testing and MIC Determination

Plates containing FLC were removed from 4° C and allowed to reach room temperature. RSA testing was initiated by inoculating wells 1-11 with 100 µl of the appropriate *C. albicans* or *C. glabrata* 0.11 OD_{530nm} yeast inoculum. Plates were sealed within tape and placed in a 35-37° C incubator for approximately 19 h. 50 µl of color mix containing 0.6 M sodium phosphate, 360 µg/ml of 4-amino antipyrine, 490 µg/ml of N-ethyl-N-sulfopropyl-m-toluidine, 0.68 U/ml horseradish peroxidase, and 0.4 U/ml glucose oxidase was added to each well. At 30 min, the color intensity was measured at OD_{550nm} with a reference reading of OD_{655nm} in a plate reader.

The published RSA protocol generates antifungal susceptibility curves by plotting $OD_{550-655nm}$ values (y-axis) against the appropriate FLC concentration (x-axis) (Fig. 6). I have modified this in our testing, for reasons detailed in the discussion, by converting $OD_{550-655nm}$ values to percent residual glucose values, which are then plotted against the appropriate FLC concentration on the x-axis. The percent residual glucose of each test well was calculated by comparing the OD_{550nm} value of the test well with the OD_{550nm} of the glucose control well based on the following equation: (OD_{550nm} of test well / OD_{550nm} of glucose control well) X 100. Another modification I have made involves the upper limit designation of the susceptibility curve's detection interval. While the original RSA protocol defines the detection interval upper limit as the $OD_{550-655nm}$ corresponding to the glucose control (i.e. 100% residual glucose), I have defined it as the percentage of

residual glucose corresponding to the highest inhibitory concentration of FLC. The lower limit definition remains the same, which is the percentage of residual glucose corresponding to the drug-free growth control. *C. albicans* and *C. glabrata* FLC MICs are designated as the lowest drug concentration in the upper 10% of the detection interval. If the residual glucose percentage value corresponding to the upper limit of the detection interval is below 80%, the FLC MIC is designated as >64 μg/ml.

NCCLS M27-A2 Assay and MIC Determination

Plates containing FLC were removed from 4° C and allowed to reach room temperature. NCCLS M27-A2 testing was initiated by inoculating wells 1-11 with 100 μl of *C. albicans*, *C. glabrata*, or *C. krusei* 1:1000 0.11 OD_{530nm} yeast inoculum. Plates were sealed within tape and placed in a 35-37° C incubator for approximately 48 h (46-52 h). Plates were removed and NCCLS 27-A2 FLC MICs determined by visual inspection using a mirror plate reader according the NCCLS M27-A2 guidelines (121), with FLC MIC endpoint values designated as the lowest FLC concentration reducing growth by 50% compared to the growth control.

C. albicans RSA FLC Testing

Results

Use of RSA Color Mix to Improve Objectivity of NCCLS M27-A2 FLC MICs

The visible growth of each *C. albicans* clinical strain immediately after the NCCLS M27-A2 48 h incubation is shown in Figures 49A and 50A. According to NCCLS M27-A2

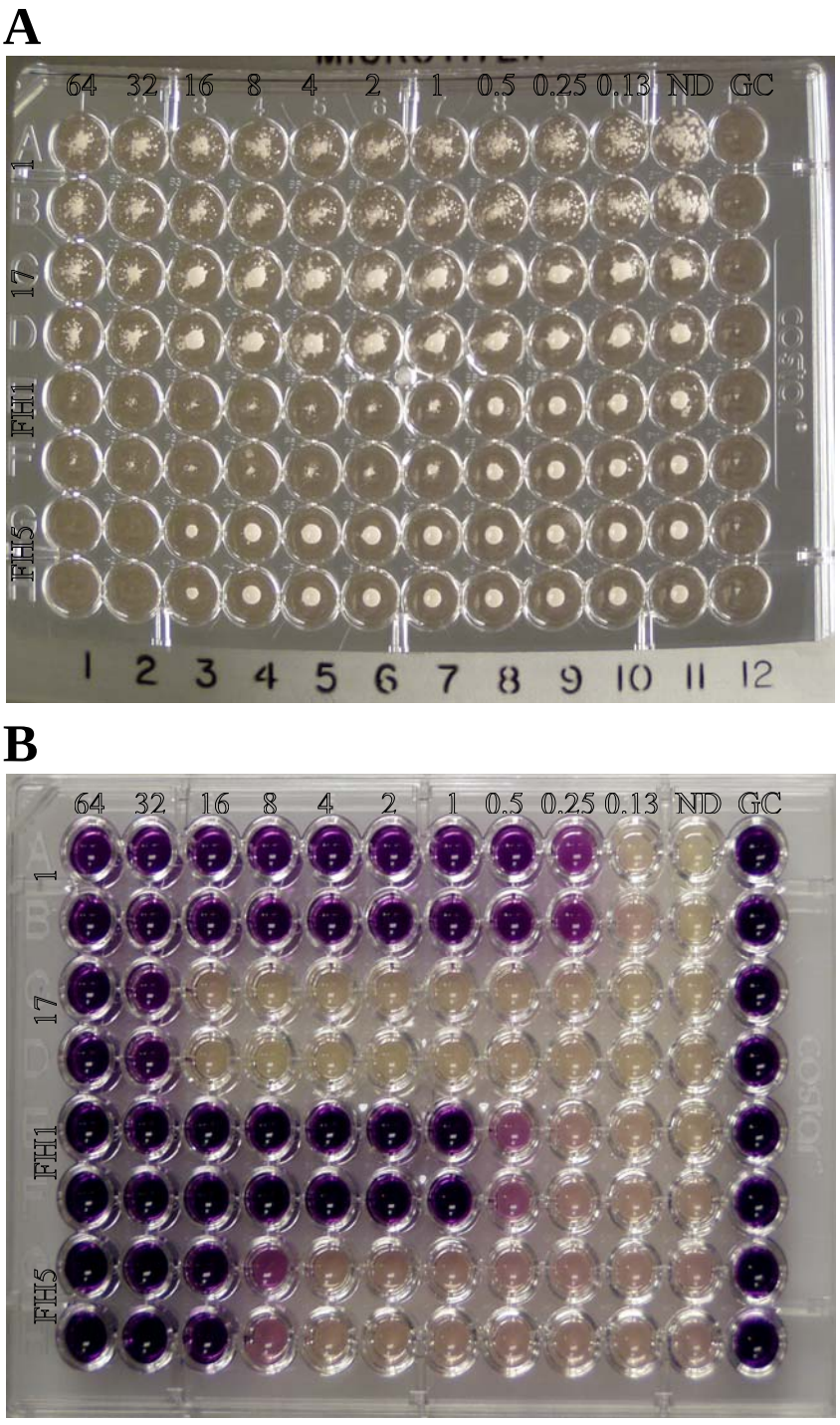


Figure 49. 48 h NCCLS M27-A2 microwell testing plate before (A) and 30 min after (B) addition of color mix. *C. albicans* strains 1, 17, FH1, and FH5 (indicated on the left vertical axis of A and B) were tested in duplicate by the NCCLS M27-A2 assay to determine their sensitivity to FLC (0.25-64 µg/ml FLC series indicated on the top horizontal axis of A and B). ND, No Drug. GC, Glucose Control.

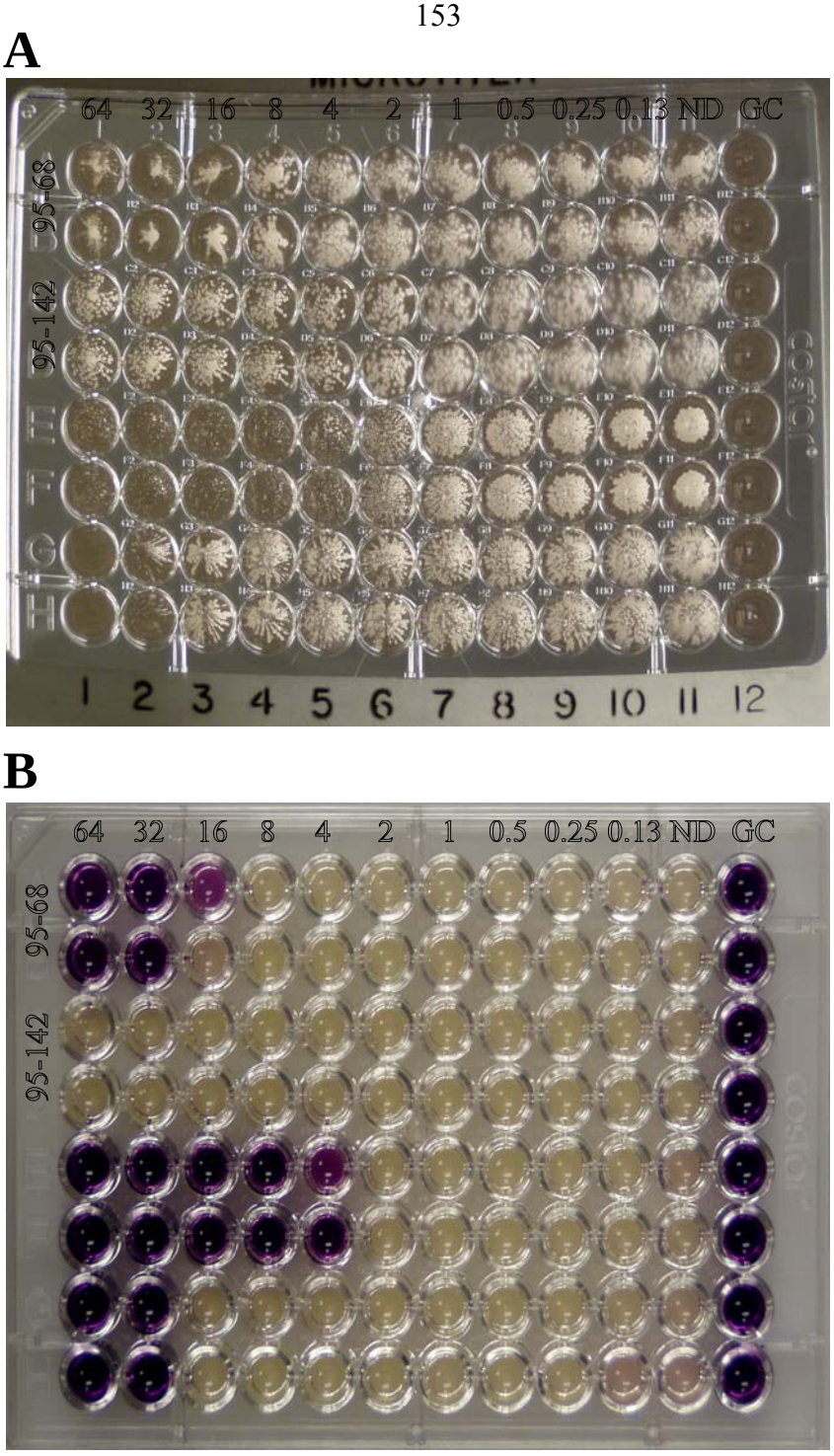


Figure 50. 48 h NCCLS M27-A2 microwell testing plate before (A) and 30 min after (B) addition of color mix. *C. albicans* strains 95-68 and 95-142 (indicated on the left vertical axis of A and B) were tested in duplicate by the NCCLS M27-A2 assay to determine their sensitivity to FLC (0.25-64 µg/ml FLC series indicated on the top horizontal axis of A and B). ND, No Drug. GC, Glucose Control.

recommended guidelines, FLC MICs were designated as the lowest drug concentration resulting in a 50% reduction in growth compared to the drug-free growth control. Trailing growth did not interfere in the determination of FLC MICs with the exception of one strain, *C. albicans* 1, who has a published NCCLS M27 FLC MIC of 0.25 μg/ml (269), but appeared to have a FLC MIC of >64 μg/ml in our NCCLS M27-A2 testing (Fig. 49A). This was resolved by adding RSA color mix to the testing wells, which facilitated a visual MIC designation of 0.5 μg/ml (Fig. 49B).

RSA FLC Testing of *C. albicans* Clinical Control Strains 19 h RSA testing of the 1:1000 OD_{530nm} yeast inocula of each *C. albicans* strain resulted in susceptibility curves that reflected the reported FLC susceptibility of each strain (Fig. 51). The susceptibility curves of FLC-sensitive *C. albicans* strains 1 and FH1 (Fig. 51A and B, respectively) had a well defined upper plateau representing the inhibitory concentrations of drug and a steep decline representing the dose-dependent inhibitory range. The susceptibility curves of FLC-resistant *C. albicans* strains FH5 and 95-68 (Fig. 51D and E, respectively) had well defined upper plateaus, steep dose-dependent inhibitory ranges, and lower plateaus, representing non-inhibitory concentrations of FLC. The susceptibility curve of the FLC-resistant *C. albicans* strain 17 (Fig. 51B) was composed of a steep dose-dependent inhibitory range and well defined lower plateau. The FLC-resistant strain 95-142 (Fig. 51F) did not form a well-defined susceptibility curve, with all of its residual glucose values falling below 80%. The lowest drug concentration corresponding to a residual glucose percentage in the top 10% percent of the detection interval was designated as the FLC MIC (Table 19). The FLC MIC of *C. albicans*

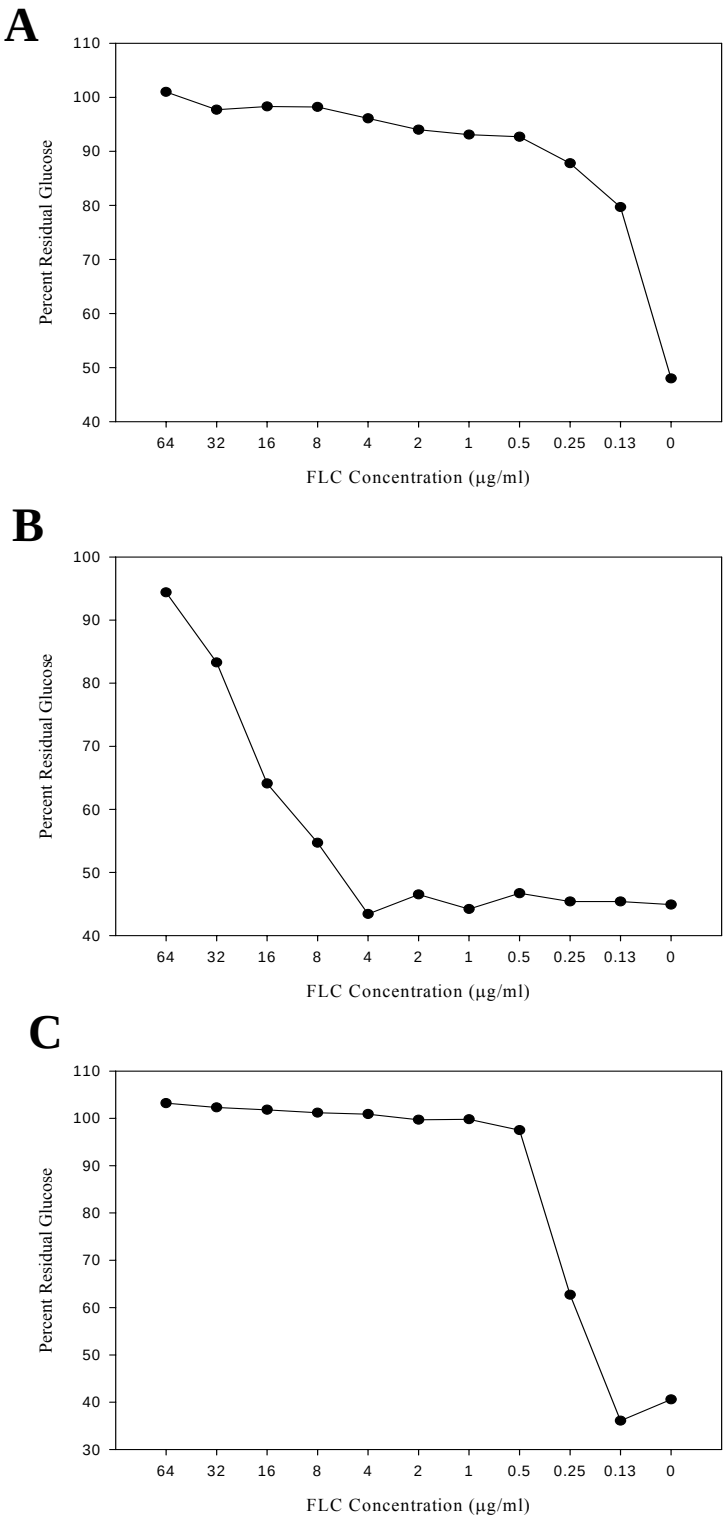


Figure 51. RSA FLC susceptibility curves of *C. albicans* 1 (A), 17 (B), FH1 (C), FH5 (D), 95-68 (E), and 95-142 (F).

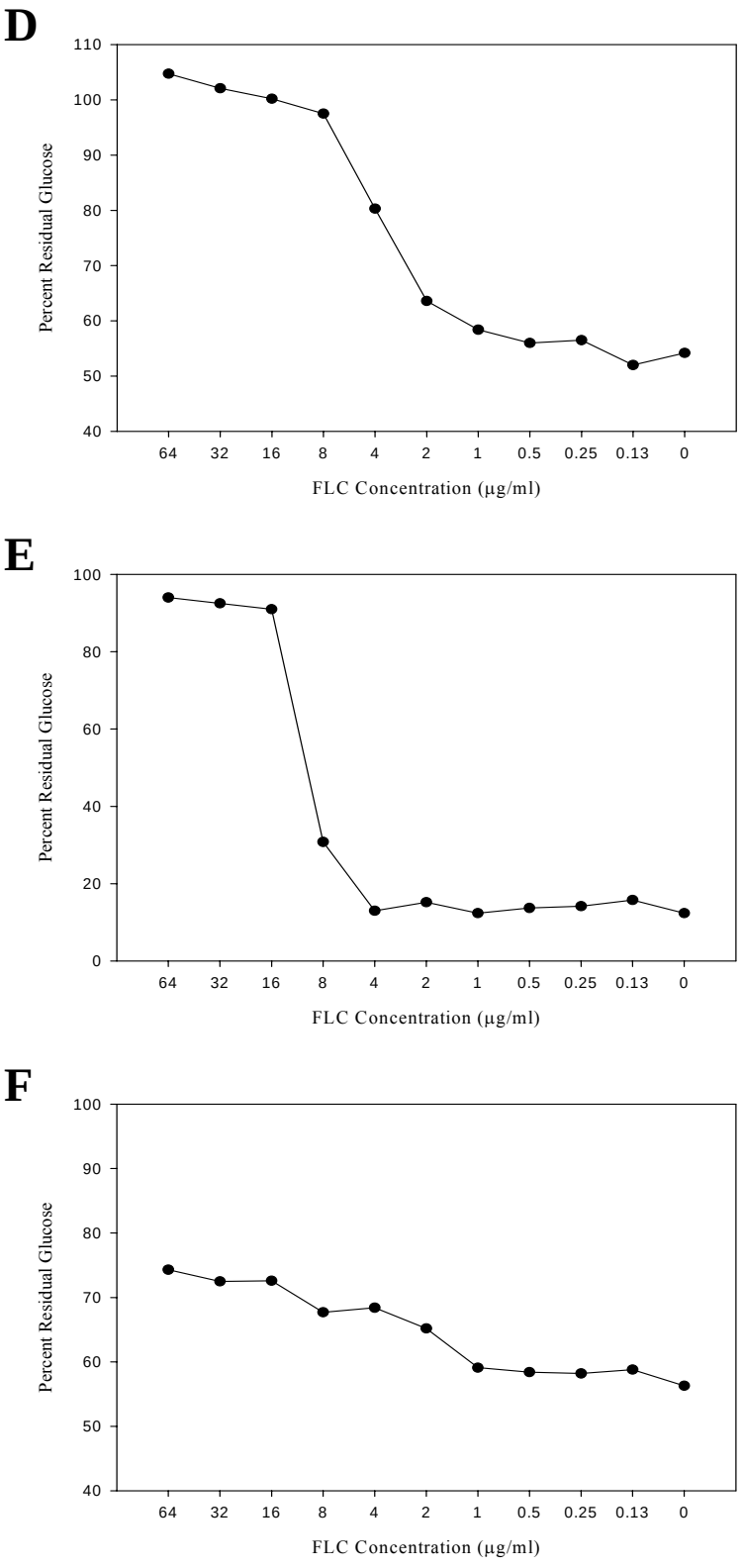


Figure 51. continued.

95-142 was arbitrarily designated as >64 µg/ml, because it did not produce a discernible susceptibility curve and the residual glucose value corresponding to 64 µg/ml was less than 80%.

Comparison of RSA and NCCLS M27-A2 FLC MICs RSA and NCCLS M27-A2 FLC MICs are listed in Table 19 (267, 269). All RSA MICs were equal to or within one two-fold dilution of those obtained by NCCLS M27-A2 testing in our laboratory. The majority of NCCLS M27-A2 FLC MICs determined in our laboratory differed from published NCCLS M27 FLC MICs by one or two two-fold dilutions. This discrepancy was not due to improper NCCLS M27-A2 testing, as our drug control, *C. krusei* strain ATCC 6258, consistently produced FLC MICs within the FLC MIC range recommended by NCCLS M27-A2 guidelines (135) (data not shown).

Table 19. 19 h RSA and 48 h NCCLS M27-A FLC MICs of *C. albicans* clinical control strains.

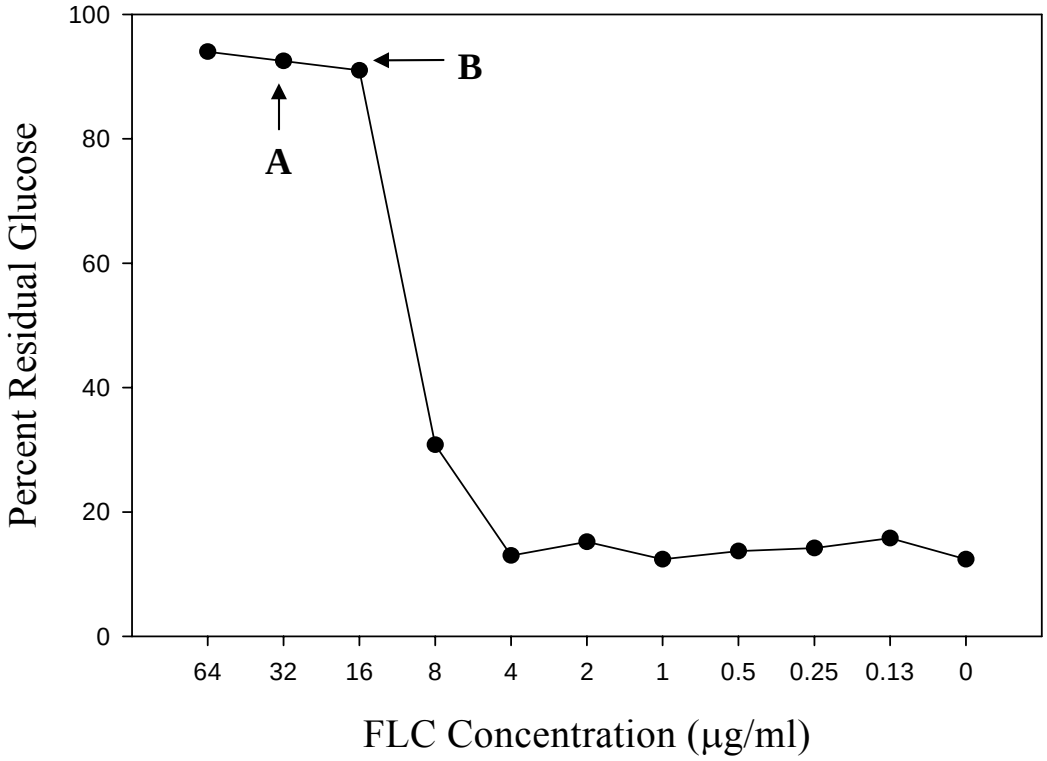
C. albicans strain	Published NCCLS M27 FLC MIC[a]	NCCLS M27-A2 FLC MIC[b]	RSA FLC MIC
1	0.25 µg/ml	0.5	0.5 µg/ml
17	≥64	64	64
FH1	4	1	0.5
FH5	≥64	32	8
95-68	≥64	16	16
95-142	≥64	>64	>64

[a] Data obtained from references 128 and 498.
[b] NCCLS M27-A2 FLC MICs determined in our laboratory.

Discussion

The goal of this research was to validate in vitro RSA FLC MICs as predictors of clinical outcome. RSA testing conditions were originally optimized using NCCLS M27-A reference strains that lacked clinical outcome data. While the use of these strains was critical in identifying testing conditions that produced highly reproducible MICs in 19 h that were equal or within one two-fold dilution of published 48 h NCCLS M27-A MICs, it did not guarantee that the RSA FLC MICs were clinically relevant. The only way to demonstrate this is by performing RSA testing with FLC-sensitive and –resistant *C. albicans* clinical control strains, isolates obtained from patients with known clinical outcomes.

Modifications to the RSA protocol were necessary to address inconsistencies in the designation of FLC MICs. The first modification was to normalize the raw residual glucose $OD_{550\text{-}655nm}$ data by converting them to percent residual glucose values. This was necessary as the $OD_{550\text{-}655nm}$ values in the upper plateaus of the susceptibility curves were not consistent, making it difficult to assign an $OD_{550\text{-}655nm}$ value cutoff that could be used to arbitrarily assign a FLC MIC of >64 μg/ml in the presence of FLC-resistant lacking a well-defined susceptibility curve. By normalizing the data, I was able to set this cut-off at 80%, which was assigned based on the percent residual glucose values of the FLC-resistant *C. albicans* strain 95-142 (Fig. 51F), which were consistently under 80%. The other modification was changing the detection interval upper limit from the residual glucose level in the glucose control well (100%) to the residual glucose level in well



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Figure 52. Comparison of FLC MICs using different definitions of the detection interval's upper limit. Upper limit definitions were either 100% residual glucose or the residual glucose percentage corresponding to 64 µg/ml FLC, which when used to determine FLC MICs, resulted in FLC MICs of 32 µg/ml (A) and 16 µg/ml (B), respectively.

these upper limit definitions can alter the resulting FLC MIC for *C. albicans* 95-68. The susceptibility curve is well defined with three inhibitory FLC concentrations in the upper plateau. If the original upper limit definition of 100% was used, the FLC was 32 µg/ml, thus accounting for only two, not three, of the inhibitory FLC concentrations. However, use of the modified upper limit definition permitted the correct MIC designation of 16 µg/ml. A 1:1000 OD_{530nm} yeast inoculum concentration was arbitrarily chosen because it

was in the midpoint of the RSA recommended FLC inoculum concentration range for *C. albicans* testing (1:512 – 1:2048) (145), but also for convenience, as this was the same inoculum concentration used in M27-A2 testing (135). The use of this yeast inoculum concentration in the RSA FLC testing of *C. albicans* clinical control strains resulted in susceptibility curves (Fig. 51) and FLC MICs (Table 19) that reflected each strain's sensitivity to FLC in vivo. In addition, RSA MICs were equal to or within one two-fold dilution of NCCLS M27-A2 FLC MICs determined in our laboratory. These data validate that in vitro RSA FLC MICs are predictors of in vivo clinical outcome. Differences between the published NCCLS M27-A2 MICs and those obtained in our laboratory, especially *with C. albicans* FH1 and 95-68, are most likely due to the extensive transferring and storage of the strains prior to us receiving the strains (personal communication with Dr. Spencer Redding). However, as the RSA and NCCLS M27-A2 FLC MIC of every strain tested in our laboratory reflected their documented in vivo sensitivities to FLC, I am confident that discrepancy is not significant.

This research has also shown that addition of RSA color mix to NCCLS M27-A2 plates after 48 h incubation can help negate the complicating effect of trailing growth on FLC MIC determination (Fig. 49, 50). The FLC-sensitive C. albicans strain 1 had a visual MIC of 0.25 µg/ml after 24 h of incubation (data not shown), which increased to >64 µg/ml after 48 h incubation (Fig. 49A). According to the NCCLS M27-A2 publication, this occurs in 5% of isolates, 24 h MICs being more clinically relevant (135). Because of this, the NCCLS M27-A2 publication now recommends that MICs be determined at 24 and 48h. As this increases the NCCLS M27-A2 workload it therefore

seemed beneficial to find a way to counteract the negative effects of trailing growth on MIC determination at 48 h. By adding the RSA color mix to testing wells of *C. albicans* 1 and allowing 30 min for color development, I was able to objectively determine a FLC MIC of 0.25 µg/ml. Visual assessment of the color reaction in testing wells of the remaining *C. albicans* strains, which did not have significant trailing growth, were also within one two-fold dilution of the FLC MIC designations made prior to addition of color mix (Table 19).

C. glabrata RSA FLC Testing

Results

Optimal Yeast Inoculum Concentration The optimal yeast inoculum concentration was determined by performing 19 h RSA testing with 1:2, 1:8, 1:32, 1:128, 1:512, 1:1000, or 1:2048 dilutions of the 0.11 OD_{530nm} yeast inocula of FLC- sensitive (6999 and 6938) and FLC–resistant (6955 and 7048) strains (Table 20). The 1:2 dilution was too concentrated as the FLC MIC of 6999, which is FLC-sensitive, was >64 µg/ml, indicating FLC resistance. In contrast, the 1:512, 1:1000, and 1:2048 dilutions were considered too dilute as the FLC MICs of 7048, FLC-resistant, were ≤2 µg/ml, indicating FLC sensitivity. Although the 1:8 and 1:32 susceptibility curves of the two FLC-sensitive strains, 6938 and 6999 (Fig. 53A and B, respectively), were well defined, residual glucose values in the upper plateau were not consistently greater than 80%, resulting FLC MIC designations of >64 µg/ml. The optimal 0.11 OD_{530nm} yeast inoculum dilution was designated as 1:128 because RSA FLC MICs were equal to or

within one two-fold of NCCLS M27-A2 FLC MICs and the susceptibility curves of the FLC-sensitive strains were well-defined, with residual glucose levels >80% in the upper plateau (Fig. 53).

Table 20. RSA FLC MIC Ranges at 19 h and NCCLS M27-A2[a] FLC MICs at 48 h

C. glabrata strain	0.11 OD_{530nm} dilution	RSA FLC MIC Range	NCCLS M27-A2 FLC MIC
6999	1:2	>64 µg/ml	
	1:8	>64	
	1:32	8	
	1:128	4	
	1:512	≤2	
	1:1000	≤2	4 µg/ml
	1:2048	≤2	
7048	1:2	≥64	
	1:8	≥64	
	1:32	64	
	1:128	16-32	
	1:512	≤2	
	1:1000	≤2	32
	1:2048	≤2	
6938	1:8	>64	
	1:32	>64	
	1:128	4	
	1:1000	NT[c]	4
6955	1:8	>64	
	1:32	64	
	1:128	64	
	1:1000	NT	64

[a] NCCLS M27-A2 testing performed in our laboratory.

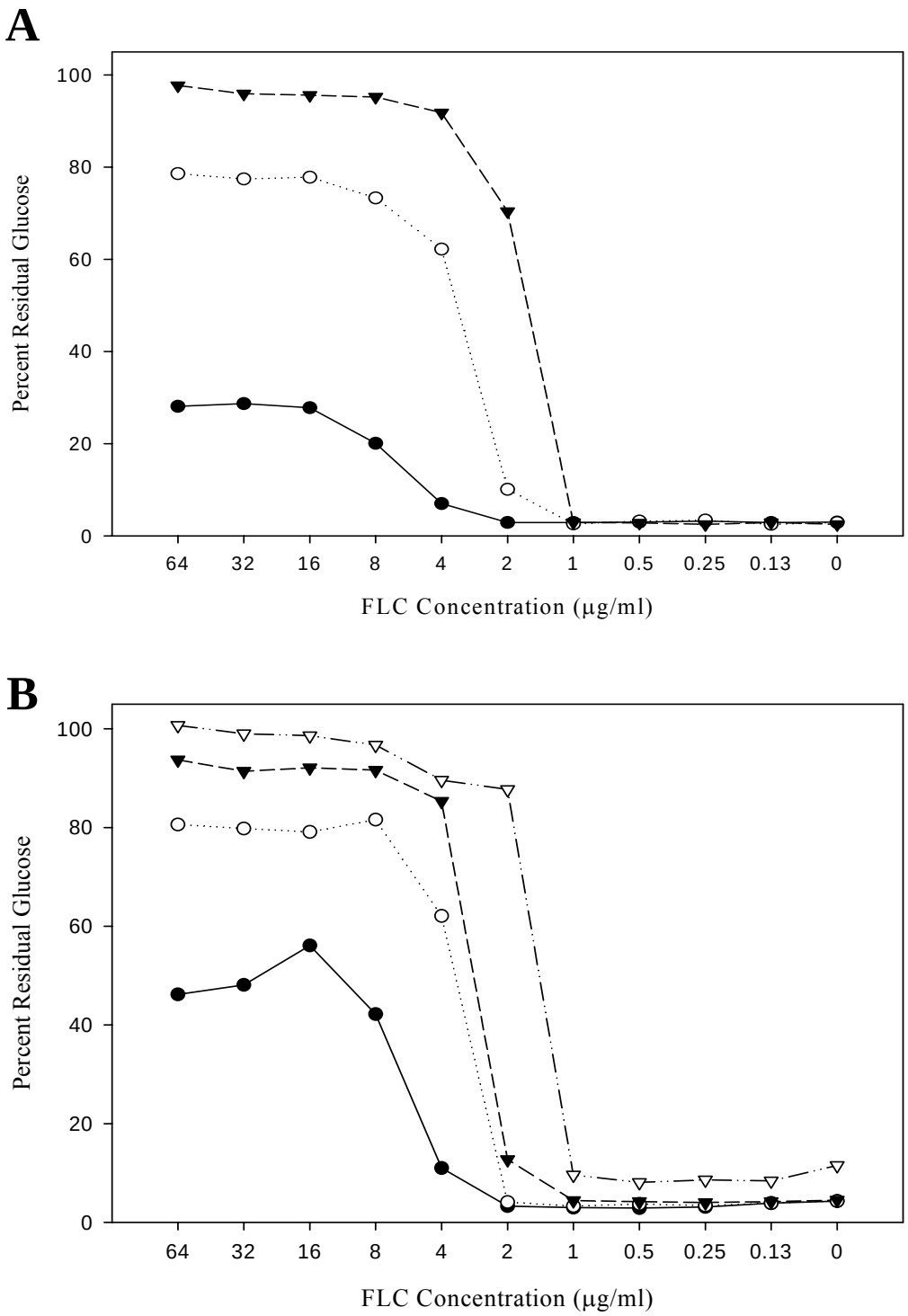


Figure 53. Susceptibility curves corresponding to the 1:8 (●), 1:32 (○), 1:128 (▼), and 1:1000 (▽) 0. 11 OD_{530nm} yeast inocula of FLC-resistant *C. glabrata* strains 6938 (A) and 6999 (B).

RSA FLC Testing of Additional *C. glabrata* isolates and Comparison to NCCLS M27-A2 FLC MICs *C. glabrata* strains 6844, 6856, 7015, and 6318 were tested in the RSA using the optimized yeast inoculum concentration of 1:128 0.11 OD_{530nm} (Table 21). All RSA FLC MICs were equal to or within one two-fold dilution of NCCLS M27-A2 MICs determined in our laboratory. The NCCLS M27-A2 MICs determined in our laboratory were not consistent with their reported MIC values (271). In the case

Table 21. *C. glabrata* 19 h RSA and 48 h NCCLS M27-A2 FLC MICs[a]

C. glabrata strain	Reported NCCLS M27 FLC MIC[b]	NCCLS M27-A2 FLC MIC[c]	RSA FLC MIC
6838	8 μg/ml	4 μg/ml	4 μg/ml
6844	8	4	4
6856	8	2	2
6955	64	32	64
6999	8	4	4
7015	16	8	8
7048	64	32	32
6318	8	16	8

[a] Testing performed three times in duplicate. MICs shown represent the geometric mean.
[b] FLC MICs according to personal communication with Spencer Redding and reference (464).
[c] NCCLS M27-A2 testing performed in our laboratory.

of *C. glabrata* 6856, there was a four-fold different between the in-house and reported MICs. As with *C. albicans* testing, I verified that our NCCLS M27-A2 testing was performed correctly by simultaneously testing the drug control strain *C. krusei* 6258, whose resulting FLC MIC was within the NCCLS M27-A2 recommended FLC MIC range (data not shown) (135).

Discussion

The goal of these experiments was to optimize RSA conditions to facilitate determination of clinically relevant *C. glabrata* FLC MICs. To accomplish this, I obtained a set of *C. glabrata* clinical control isolates, with previously known or published patient clinical outcomes and/or NCCLS M27-A2 FLC MICs (personal communication with Dr. Spencer Redding), to optimize RSA conditions, thus ensuring that the MICs are predictive of clinical outcome.

As discussed earlier, the RSA protocol was modified to address inconsistencies in *C. albicans* and *C. glabrata* MIC determination. As with our *C. albicans* RSA testing, the $OD_{550\text{-}655nm}$ data corresponding to the upper plateau of the susceptibility curve differed between both in replicate testing of each strain and between strains, making designation of an arbitrary $OD_{550\text{-}655nm}$ cutoff, for designating the FLC MIC of highly FLC-resistant strains as >64 μg/ml, difficult. By converting raw $OD_{550\text{-}655nm}$ data to percent residual glucose I was able to determine that inhibitory concentrations of FLC in the upper plateau of the 1:128 0.11 OD_{530nm} susceptibility curves of FLC-sensitive *C. glabrata* strains 6938 and 6999 were consistently greater than 80% (Figure 51), while non-inhibitory concentrations of FLC in the dose-dependent range or lower plateau of the 1:128 0.11 OD_{530nm} susceptibility curves of FLC-resistant *C. glabrata* strains 7048 and 6955 were consistently lower than 80% (data not shown). Therefore, like *C. albicans*, I was able to designate 80% residual glucose as the cutoff for an arbitrary FLC MIC designation of >64 μg/ml. I also found it necessary to change the detection interval upper limit from 100% residual glucose to the residual glucose value corresponding to 64

μg/ml. As with *C. albicans*, I found that by using the published protocol our FLC MICs were two- to four-fold greater than would be predicted based on analysis of the susceptibility curve (data not shown).

The dose-dependence effect of yeast inoculum concentration on FLC MIC was demonstrated by performing RSA testing with a range (1:2 to 1:2048) of diluted 0.11 OD_{530nm} yeast inocula of FLC-sensitive *C. glabrata* strains 6938 and 6999 and FLC-resistant *C. glabrata* strains 7048 and 6955 (Table 20). I was able to eliminate overly concentrated or dilute concentrations by comparing the FLC MICs to each strains' known in vivo susceptibility to FLC and by analyzing the corresponding susceptibility curves (Fig. 53). The 1:128 dilution was the only dilution consistently producing susceptibility curves with clinically relevant MICs. RSA FLC testing of the remaining *C. glabrata* strains, under these same conditions, also resulted in clinically relevant MICs, with all RSA FLC MICs being equal to or within one two-fold dilution of FLC obtained by NCCLS M27-A2 testing performed in our laboratory (Table 21). As mentioned before, differences between the published and in-house NCCLS M27-A2 FLC MICs are most likely due to the extensive transferring and storage time that may have occurred prior to us receiving the strains.

In conclusion I was able to optimize RSA conditions to generate clinically relevant *C. glabrata* FLC MICs. However, as *C. glabrata* infections are primarily treated with either FLC or AMB {460}, an essential task that remains undone is to locate AMB-sensitive and/or AMB-resistant *C. glabrata* clinical control strains and use them to optimize conditions for RSA AMB testing of *C. glabrata* isolates.

CHAPTER 7

CONCURRENT AND SEQUENTIAL ANTIFUNGAL (AMPHOTERICIN B, ITRACONAZOLE, AND VORICONAZOLE) COMBINATION TESTING OF CONIDIAL AND HYPHAL INOCULA OF *ASPERGILLUS FUMIGATUS* ISOLATES

Introduction

The increasing incidence of invasive aspergillosis (IA) and advent of new antifungals has generated interest in concurrent antifungal combination therapy. In, addition, the increasing use of secondary and sometimes tertiary antifungal regimens for IA, necessary due to fungal resistance to or the toxic effects of primary antifungal regimens, have prompted both physicians and investigators to question the safety and efficacy of sequential antifungal therapy.

I have utilized in vitro checkerboard testing formats to determine the effects of two-drug concurrent and sequential combinations of amphotericin B (AMB), itraconazole (ITC), or voriconazole (VRC) on the conidial and hyphal inocula of *A. fumigatus*. AMB, ITC, and VRC were chosen as they are the only three antifungal drugs currently approved for primary therapy of IA. Although I previously demonstrated that *A. fumigatus* conidia and hyphae have similar susceptibilities to AMB, ITC, and VRC (Chapter 4), I could not assume that this would also be true with antifungal combination testing. I therefore performed antifungal combination testing with both conidial and hyphal inocula. The effects of concurrent and sequential antifungal combinations were interpreted by generating fractional inhibitory concentration (FIC) index definitions that

permitted a ± 1 dilution experimental error that is often observed in checkerboard testing (183, 201).

Materials and Methods

Reagents

Preparations of RPMI 1640 with or without 0.4% glucose were buffered with 0.165 M 3-(N-morpholino)-propanesulfonic acid and adjusted to pH 7.0 with 10 N NaOH.

Fungal Strains

A. fumigatus strains NCPF 7101, 7100, 7098, and 7097 were purchased from the National Collection of Pathogenic Fungi (Mycology Reference Laboratory, Bristol). *A. fumigatus* AF-72 was donated by Dr. Denning at Hope Hospital, UK. These strains were isolated from aspergillosis patients with known clinical outcomes. Species designations of *A. fumigatus* were confirmed by observations of macroscopic and microscopic characteristics during growth on Czapek-Dox agar (35). All stocks were maintained in vials of sterile water at 4º C.

Antifungal Agents and Preparation

AMB and ITC were purchased in powdered form. VRZ was generously provided by Pfizer, Inc. Stocks of 1600 µg/ml were prepared for each drug in dimethyl sulfoxide (DMSO) and stored at -80°C for up to 6 months. 41x AMB, ITC, and VRC twofold dilution series' were prepared in DMSO for cRSA testing of each *A. fumigatus* isolate

(Table 22). The 41x drug series' were stored at -80º C overnight and warmed to room temperature before use.

Table 22. Final AMB, ITC, and VRC ranges in antifungal combination testing of *A. fumigatus* isolates[a]

A. fumigatus isolate	AMB range (µg/ml)	ITC range (µg/ml)	VRC range (µg/ml)
NCPF 7097	0.13 – 8.0	0.06 – 4.0	0.06 – 4.0
NCPF 7098	0.06 – 4.0	0.06 – 4.0	0.06 – 4.0
NCPF 7100	0.06 – 4.0	0.13 – 8.0	0.13 – 8.0
NCPF 7101	0.06 – 4.0	0.06 – 4.0	0.06 – 4.0

[a] Final drug range concentrations after adding 5µl of 41x drug range to testing and control wells containing 200 µl RPMI.

Microwell Plate Preparation.

Each cRSA plate was prepared by inoculating 100µl of 0.4% glucose RPMI into the first 8x8 grid of wells and the last column of wells in a 96-well microtiter plate. Plates were sealed and stored at 4º C until use.

Conidial Inoculum Preparation Isolates were cultured on Sabouraud glucose agar (SAB) (BBL, Cockeysville, MD, cat. no. 211584) slants at 30º C for 3 days. After flooding with glucose deficient RPMI 1640, the slant surface was gently scraped with a sterile wooden applicator stick. Once the hyphal fragments were allowed to settle for approximately 15 min, the conidial suspension was collected and adjusted with glucose deficient RPMI 1640 to an optical

density (OD_{530nm}) of 0.11, then diluted 1:50. Viability of each conidial suspension was determined by plating 0.1ml of the 0.11 OD_{530nm} conidial inoculum, diluted 1:10,000, on SAB plates and incubating at 37º C.

Concurrent Antifungal Combination Testing

Microtiter plates containing 0.4% glucose RPMI were removed from 4º C and allowed to reach room temperature. 100 µl of 1:50 0.11 OD_{530nm} conidial inoculum was inoculated into the 8x8 testing well grid. Wells in the last column were inoculated with

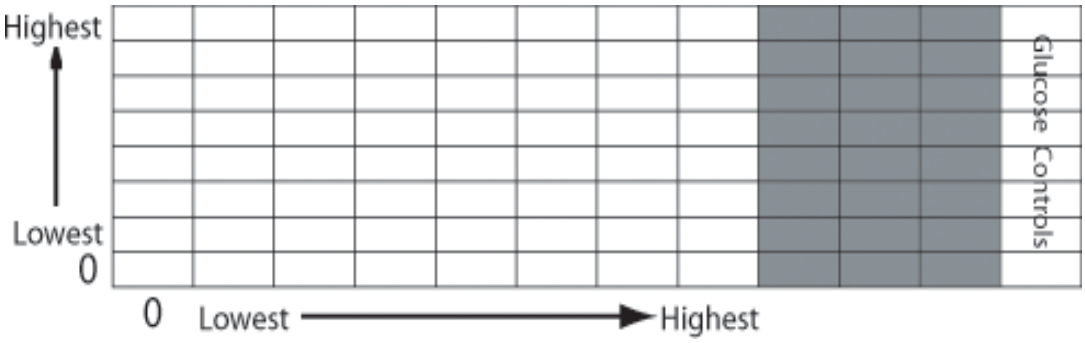


Figure 54. Illustration of antifungal drug checkerboard set-up in a testing plate. For the first drug, 5 µl of DMSO was added to column 1 while 5 µl of drug was added to columns 2 – 8, with drug concentrations increasing from the left to the right. For the second drug, 5 µl of DMSO was added to row 8 while 5 µl of drug was added to rows 1 – 7, with drug concentrations increasing from the bottom to the top. The last column (glucose controls) was inoculated with 10 µl of DMSO.

100 µl of glucose-deficient RPMI (glucose controls). Hyphae plates were sealed and incubated at 35-37º C for 12 h before proceeding to the next step. Concurrent antifungal susceptibility testing was initiated in both conidial and hyphal plates by inoculating testing wells with 5µl of two of the 41x AMB, ITC, or VRZ dilution ranges or DMSO

(drug-free testing wells and glucose control wells) (Fig. 54). Plates were sealed with tape and shaken at 150 rpm for 15 min. After incubating at 35-37º C for 48 h, plates were removed and fungal growth assessed by visual inspection using a mirror plate reader. 50µl of the enzyme color mix (same composition as in mRSA testing) was then added to each well. At 20 min, color intensity was measured at OD_{550nm} with a reference reading of OD_{655nm} in a plate reader.

Sequential Antifungal Combination Testing

Microtiter plates containing 0.4% glucose RPMI were removed from 4º C and allowed to reach room temperature. 100 µl of 1:50 0.11 OD_{530nm} conidial inoculum was inoculated into the 8x8 testing well grid. Wells in the last column were inoculated with 100 µl of glucose-deficient RPMI (glucose controls). Hyphae plates were sealed and incubated at 35-37º C for 12 h before proceeding to the next step. Sequential antifungal combination testing was initiated in both conidial and hyphal plates by inoculating testing wells with 5 µl of one of the 41x AMB, ITC, or VRZ dilution ranges or DMSO (drug-free testing wells and glucose control wells). Plates were sealed with tape and shaken at 150 rpm for 15 min. After incubating at 35-37º C for 8h, plates were removed and 5 µl of the second 41x drug dilution range or DMSO (drug-free testing wells and glucose control wells) were added to the wells. Plates were sealed with tape and shaken at 150 rpm for 15 min. After incubating at 35-37º C for 48 h, plates were removed and fungal growth assessed by visual inspection using a mirror plate reader.

Analysis of Drug Interactions

Drug interactions were analyzed by a fractional inhibitory concentration (FIC) index method. Like the NCCLS M38-A assay, AMB, ITC, and VRC MIC endpoint values were based on visual determination of inhibition of growth. FIC indices in concurrent and sequential antifungal combination testing of conidial inocula were determined based on AMB, ITC, and VRC MIC endpoint definitions of 100% growth inhibition. FIC indices in concurrent and sequential antifungal combination testing of hyphal inocula were determined based on AMB, ITC, and VRC MIC endpoint definitions of 80% growth inhibition. For purposes of calculation, off-scale MICs were converted to the next higher dilution. The FIC of each drug was calculated as the ratio of the concentration of the drug in combination that achieves the MIC endpoint to the MIC of the drug alone by using that endpoint. FIC index values were calculated by adding the individual FIC values of each drug present in the well. FIC index values were interpreted as follows: ≤ 0.5, synergistic; $> 0.5 – 1.0$, additive; $> 1.0 – 4.0$ indifferent; > 4.0, antagonistic.

Results

AMB, ITC, and VRC MICs of Conidial and Hyphal Inocula

To assess whether combination testing was prepared correctly, the monodrug AMB, ITC, and VRC MICs of conidial and hyphal conidial inocula were compared to previously determined AMB, ITC, and VRC NCCLS M38-A and hRSA MICs, respectively. The AMB, ITC, and VRC MICs corresponding to the conidial inocula of

each *A. fumigatus* strain, determined by visual inspection of fungal growth in the first column and last row of combination microwell plates, were equal to or within a two-fold dilution of NCCLS M38-A MICs (Table 23). The AMB, ITC, and VRC MICs corresponding to the hyphal inocula of each *A. fumigatus* strain, also determined visual inspection of fungal growth in the first column and last row of combination microwell plates, were equal to or within a two-fold dilution of hRSA MICs (Table 24).

Concurrent Antifungal Combination Testing of Conidial and Hyphal Inocula

The interaction effects of concurrent antifungal combinations AMB and ITC (AMB+ITC), AMB and VRC (AMB+VRC), and ITC and VRC (ITC+VRC) on conidial and hyphal inocula are shown in Tables 25 and 26, respectively. Concurrent antifungal combination testing with conidial inocula resulted in additive and/or indifferent effects, regardless of the drug combination and strain being tested. Although these antifungal combinations also resulted in additive and/or indifferent effects with hyphal inocula, synergy was also observed: AMB+ITC had a synergistic effect on the hyphal inocula of every strain; AMB+VRC had a synergistic effect on the hyphal inocula of strains NCPF 7097 and 7098; and ITC+VRC had a synergistic effect on the hyphal inoculum of strain NCPF 7100.

Sequential Antifungal Combination Testing of Conidial and Hyphal Inocula

The interaction effects of sequential antifungal combinations of AMB followed by ITC (AMB→ITC), AMB followed by VRC (AMB→VRC), ITC followed by AMB (ITC→AMB), ITC followed by VRC (ITC→VRC), VRC followed by AMB

Table 23. Comparison between AMB, ITC, and VRC MICs obtained with conidial inocula in current testing and previous NCCLS M38-A testing

Drug	Testing	MIC (μg/ml) (geometric mean)			
		NCPF 7101	NCPF 7097	NCPF 7098	NCPF 7100
AMB	Current	1-4 (2)	2	2-4 (2)	1-4 (1)
	NCCLS M38-A	1	1	1	1
ITC	Current	0.25-0.5 (0.25)	0.25-0.5 (0.25)	0.5	>8
	NCCLS M38-A	0.5	0.5	0.5	>16
VRC	Current	0.25-0.5 (0.25)	0.25-0.5 (0.25)	0.25-0.5 (0.5)	1
	NCCLS M38-A	0.25	0.25	0.5	1

Table 24. Comparison between AMB, ITC, and VRC MICs obtained with hyphal inocula in current testing and previous hRSA[a] testing

Drug	Testing	MIC (μg/ml) (geometric mean)			
		NCPF 7101	NCPF 7097	NCPF 7098	NCPF 7100
AMB	Current	0.5-1.0 (1)	1	1-2 (2)	0.25-0.5 (0.25)
	hRSA	1	4	1	0.5
ITC	Current	0.25-1 (0.25)	0.25-0.5 (0.5)	0.25-0.5 (0.5)	8->8 (>8)
	hRSA	0.25	0.5	0.25	>16
VRC	Current	0.5	0.25-1 (0.5)	0.25-0.5 (0.5)	0.5-1 (1)
	hRSA	0.5	0.5	0.5	2

[a] MICs values corresponding to hyphal inocula prepared with 1:50 0.11 OD_{530nm} conidia inocula.

(VRC→AMB), and VRC followed by ITC (VRC→ITC) on conidial and hyphal inocula are shown in Tables 27 and 28, respectively. AMB→ITC had additive and/or indifferent effects on the majority of conidial inocula with the exception of strain NCPF 7098, in which a synergistic effect was also observed. In contrast, AMB→ITC had a synergistic effect on the majority of hyphal inocula, with the exception of strain NCPF 7100,

Table 25. Concurrent interactions between AMB, ITC, and VRC determined by the FIC index (FICI) for *A. fumigatus* conidial inocula[a]

Drug Combination	FICI Range (interaction[b])			
	NCPF 7101	NCPF 7097	NCPF 7098	NCPF 7100
AMB+ITC	1.06-2.5 (I)	1.06-2.5 (I)	1.03-2.25 (I)	1.01-1.5 (I)
AMB+VRC	1-2.25 (A/I)	1-2.25 (A/I)	1.03-2.25 (I)	1-2.25 (A/I)
ITC+VRC	0.75-1.5 (A/I)	0.75-1.5 (A/I)	1-1.25 (A/I)	1-1.5 (A/I)

[a] Testing performed in duplicate or triplicate.
[b] Interaction: A, additive (FICI >0.5 – 1); I, indifferent (FICI >1 – 4).

Table 26. Concurrent interactions between AMB, ITC, and VRC determined by the FIC index (FICI) for *A. fumigatus* hyphal inocula[a]

Drug Combination	FICI Range (interaction[b])			
	NCPF 7101	NCPF 7097	NCPF 7098	NCPF 7100
AMB+ITC	0.38-1.13 (S/A/I)	0.38-1.13 (S/A/I)	0.26-0.38 (S)	0.38-1.02 (S/A/I)
AMB+VRC	0.62-2.25 (A/I)	0.5-1.25 (S/A/I)	0.5-1.13 (S/A/I)	1.13-2 (I)
ITC+VRC	1-1.5 (A/I)	1-1.5 (A/I)	1-1.5 (A/I)	0.38-1.13 (S/A/I)

[a] Testing performed in duplicate or triplicate.
[b] Interaction: S, synergy (FICI ≤0.5); A, additive (FICI >0.5 – 1); I, indifferent (FICI >1 – 4).

in which only additive and indifferent effects were observed. In addition to additive and indifferent effects, AMB→VRC had synergistic effects on the conidial inocula of two of the strains, NCPF 7097 and 7100, but a synergistic effect on the hyphal inoculum of only one strain, NCPF 7100. While ITC→AMB and VRZ→AMB had additive and/or indifferent effects on both conidial and hyphal inocula, they also consistently resulted in

Table 27. Sequential interactions between AMB, ITC, and VRC determined by the FIC index (FICI) for *A. fumigatus* conidial inocula[a]

Drug Combination	FICI Range (interaction[b])			
	NCPF 7101	NCPF 7097	NCPF 7098	NCPF 7100
AMB→ITC	1-2.25 (A/I)	1-2.5 (A/I)	0.5-1.25 (S/A/I)	1.01-1.5 (I)
AMB→VRC	0.75-1.25 (A/I)	0.5-1.25 (S/A/I)	0.75-2.06 (A/I)	0.5-1.25 (S/A/I)
ITC→AMB	1.13-16.5 (I/T)	1.06-8.5 (I/T)	2.03-4.5 (I/T)	8.01-9 (T)
ITC→VRC	1.13-2 (I)	1-1.5 (A/I)	0.63-1.25 (A/I)	0.63-1.13 (A/I)
VRC→AMB	0.75-4.25 (A/I/T)	0.75-4.25 (A/I/T)	1-4.5 (A/I/T)	1.03-5 (I/T)
VRC→ITC	1-1.25 (A/I)	1.25-2.5 (I)	0.63-2.25 (A/I)	1.01-2 (I)

[a] Testing performed in duplicate or triplicate.
[b] Interaction: S, synergy (FICI ≤0.5); A, additive (FICI >0.5 – 1); I, indifferent (FICI >1 – 4); T, antagonism (FICI >4) .

Table 28. Sequential interactions between AMB, ITC, and VRC determined by the FIC index (FICI) for *A. fumigatus* hyphal inocula[a]

Drug Combination	FICI Range (interaction[b])			
	NCPF 7101	NCPF 7097	NCPF 7098	NCPF 7100
AMB→ITC	0.5-0.75 (S/A)	0.38-0.75 (S/A)	0.38-2.06 (S/A/I)	0.52-1.25 (A/I)
AMB→VRC	0.56-1.25 (A/I)	0.75-2.25 (A/I)	0.63-1.25 (A/I)	0.5-1.25 (S/A/I)
ITC→AMB	1.06-9 (I/T)	1.06-9 (I/T)	1.03-6 (I/T)	4.01-4.5 (T)
ITC→VRC	1-2.25 (A/I)	0.75-1.25 (A/I)	1.13-2 (I)	0.63-1.25 (I)
VRC→AMB	1.03-5 (I/T)	2.13-16.5 (I/T)	4.03-6 (T)	1.25-33 (I/T)
VRC→ITC	1.13-2 (I)	0.63-1.25 (A/I)	1-1.5 (A/I)	1-1.03 (A/I)

[a] Testing performed in duplicate or triplicate.
[b] Interaction: S, synergy (FICI ≤0.5); A, additive (FICI >0.5 – 1); I, indifferent (FICI >1 – 4); T, antagonism (FICI >4).

antagonistic effects. ITC→VRC and VRC→ITC resulted in additive and/or indifference effects on the conidial and hyphal inocula of all strains.

Discussion

The goals of this study were to determine the effects of concurrent and sequential two-drug combinations of AMB, ITC, or VRC on the conidial and hyphal inocula of *A. fumigatus* strains NCPF 7097, 7098, 7100, and 7101 and to ascertain whether these drug combinations had differential effects on conidia versus hyphae. To our knowledge, this is the first study that has compared the in vitro sensitivities of *A. fumigatus* conidia and hyphae to concurrent and sequential combinations of antifungal drugs.

In vitro combination testing can be performed in several formats including the time-kill, checkerboard, and most recently, E-test format. As our testing included more than seventy concurrent and sequential antifungal combinations, I chose the checkerboard format as it is more convenient than the time-kill format and has been more extensively used than the E-test format. Each two-drug checkerboard combination test was performed in a 96-well plate, with testing wells comprising an 8x8 grid of testing wells (Fig. 54). This design facilitated MIC determinations with each drug alone as well as testing of 49 concentration combinations of the two drugs per plate.

Antifungal combination testing of conidial and hyphal inocula required different testing parameters. Preliminary conidial inocula concurrent combination testing performed in 16 h, using mRSA testing parameters, and 48 h, using NCCLS M38-A testing parameters, demonstrated that reproducible drug interaction results could be

determined in 48 h, but not 16 h (data not shown). As a result, all subsequent concurrent and sequential combination testing of conidial inocula were performed using the NCCLS M38-A testing parameters of conidial inoculum concentration (1:50 0.11 OD_{530nm}), glucose concentration (0.2%), and MIC endpoint definitions (100% visible growth inhibition). Concurrent and sequential combination testing of conidial inocula were prepared correctly as the monodrug AMB, ITC, and VRC MICs corresponding to conidial inocula in each combination plate after 48 (concurrent testing) or 54 h (sequential testing) were equal to or within a two-fold dilution of AMB, ITC, and VRC MICs determined in previous 48 h NCCLS M38-A testing (Table 23). In contrast, testing parameters for concurrent and sequential combination testing of hyphal inocula were based on those used in hRSA testing (Chapter 4) with the exception of the MIC endpoint definition used in combination testing, which was a visible 80% inhibition of growth. This endpoint was necessary due to fungal growth for 12 h prior to addition of both drugs in concurrent antifungal testing and 20 h of fungal growth prior to addition of the second drug in sequential antifungal testing. Concurrent and sequential combination testing of hyphal inocula were prepared correctly as the monodrug AMB, ITC, and VRC MICs corresponding to hyphal inocula in each combination plate after 60 or 68 h were equal to or within a two-fold dilution of the AMB, ITC, and VRC MICs determined in previous 60 h hRSA testing (Table 24).

Although not an issue in concurrent and sequential antifungal combination testing with conidial inocula, the growth-based MIC endpoint definition of 80% growth reduction used in determining FIC indices in concurrent and sequential antifungal

combination testing of hyphal inocula introduced subjectivity to our interpretations of combinatorial antifungal effects. Unfortunately, the use of a growth-based MIC endpoint definition was necessary as comprehensive RSA-based endpoint definitions could not be established for antifungal combination testing of conidial or hyphal inocula and response surface statistical analysis did not consistently produce drug interaction values (ICα) that reflected the true drug interaction effect. RSA-based MIC endpoint definitions could not be established because an appropriate detection interval cut-off value could not be determined. For example, when the conidial inoculum of *A. fumigatus* NCPF 7097 was incubated in the presence of ITC for 8 h prior to addition of AMB, visible antagonistic effects were observed after 48 h incubation (Fig. 67B). Figure 55 shows how the use of a 20, 30, 40 and 50% (Fig. 55A, B, C, and D, respectively) RSA detection interval cut-off value affected the FIC index determination. The use of 20, 30, or 40% detection interval cutoff values was unsuitable because artificially high FIC indices were generated. The use of a 50% detection interval cutoff was suitable as the resulting indices were identical to those determined by the use of subjective (visual-based) MIC endpoint definitions. Unfortunately, a 50% detection interval cutoff was not sufficient in determining FIC indices on all plates. When the conidial inoculum of *A. fumigatus* NCPF 7097 was incubated in the presence of VRC and AMB, additive and/or indifferent effects were visually observed after 48 h incubation (Fig. 59B). Figure 56 shows how the use of a 40, 50, 60 and 90% (Fig. 56A, B, C, and D, respectively) RSA detection interval cut-off value affected the FIC index determination. Surprisingly, the use of all of the detection interval cutoff values generated artificially high FIC indices. The use of a RSA detection

cutoff of > 90% would in fact be unsuitable as its use in the first example (I→A) would result in a drug interaction interpretation of indifference instead of antagonism (data not shown). I also found that the objective data generated by the RSA could not be used in the Greco response surface statistical analysis program, ModLab, as imputing RSA data from one of the NCPF 7097 I→A conidial inoculum testing plates, in which antagonism was visibly observed, into the program resulted in an interaction coefficient alpha value that indicated synergy (data not shown).

Our interpretations of the in vitro effects of concurrent and sequential antifungal combinations on the conidial and hyphal inocula of *A. fumigatus* strains NCPF 7101, 7098, and 7097 generally reflected the in vivo effects of concurrent and sequential antifungal therapies used in treating invasive aspergillosis (IA) patients. While AMB+ITC therapy has been reported as having both synergistic and antagonistic effects on IA outcome (207), I found that the AMB+ITC consistently had an indifferent effect on conidial inocula but a synergistic effect on hyphal inocula. Although I was unable to find any reports of AMB+VRC therapy, I found that the effect of AMB+VRC was similar to AMB+ITC, except that synergy was only observed with the hyphal inocula of *A. fumigatus* strains NCPF 7097 and 7098. I was also unable to find any reports of ITC+VRC, but as these antifungal drugs have the same target, P450 14α-demethylase, I was not surprised that their combination resulted in an additive and/or indifferent effect on both conidial and hyphal inocula. AMB→ITC and AMB→VRC therapy has been repeatedly documented as causing no harm and in certain cases being more efficacious than AMB or ITC monotherapy (209, 212). The in vitro effects of AMB→ITC and

A

82.4%	98.4%	84.8%	100.6%	86.9%	82.7%	102.2%	103.9%
103.3%	101.2%	92.8%	100.9%	92.0%	90.4%	102.4%	104.7%
99.6%	95.0%	81.9%	83.9%	82.4%	84.1%	93.2%	103.0%
103.3%	84.5%	82.8%	84.3%	83.3%	85.0%	97.8%	102.9%
99.0%	94.2%	91.5%	96.6%	84.1%	86.5%	99.9%	102.3%
85.7%	74.8%	61.5%	57.4%	54.4%	73.6%	96.0%	98.5%
66.4%	50.2%	51.9%	46.0%	40.2%	55.4%	62.0%	70.9%
59.0%	57.3%	59.2%	59.6%	55.2%	94.2%	93.4%	101.6%

B

82.4%	98.4%	84.8%	100.6%	86.9%	82.7%	102.2%	103.9%
103.3%	101.2%	92.8%	100.9%	92.0%	90.4%	102.4%	104.7%
99.6%	95.0%	81.9%	83.9%	82.4%	84.1%	93.2%	103.0%
103.3%	84.5%	82.8%	84.3%	83.3%	85.0%	97.8%	102.9%
99.0%	94.2%	91.5%	96.6%	84.1%	86.5%	99.9%	102.3%
85.7%	74.8%	61.5%	57.4%	54.4%	73.6%	96.0%	98.5%
66.4%	50.2%	51.9%	46.0%	40.2%	55.4%	62.0%	70.9%
59.0%	57.3%	59.2%	59.6%	55.2%	94.2%	93.4%	101.6%

C

82.4%	98.4%	84.8%	100.6%	86.9%	82.7%	102.2%	103.9%
103.3%	101.2%	92.8%	100.9%	92.0%	90.4%	102.4%	104.7%
99.6%	95.0%	81.9%	83.9%	82.4%	84.1%	93.2%	103.0%
103.3%	84.5%	82.8%	84.3%	83.3%	85.0%	97.8%	102.9%
99.0%	94.2%	91.5%	96.6%	84.1%	86.5%	99.9%	102.3%
85.7%	74.8%	61.5%	57.4%	54.4%	73.6%	96.0%	98.5%
66.4%	50.2%	51.9%	46.0%	40.2%	55.4%	62.0%	70.9%
59.0%	57.3%	59.2%	59.6%	55.2%	94.2%	93.4%	101.6%

D

82.4%	98.4%	84.8%	100.6%	86.9%	82.7%	102.2%	103.9%
103.3%	101.2%	92.8%	100.9%	92.0%	90.4%	102.4%	104.7%
99.6%	95.0%	81.9%	83.9%	82.4%	84.1%	93.2%	103.0%
103.3%	84.5%	82.8%	84.3%	83.3%	85.0%	97.8%	102.9%
99.0%	94.2%	91.5%	96.6%	84.1%	86.5%	99.9%	102.3%
85.7%	74.8%	61.5%	57.4%	54.4%	73.6%	96.0%	98.5%
66.4%	50.2%	51.9%	46.0%	40.2%	55.4%	62.0%	70.9%
59.0%	57.3%	59.2%	59.6%	55.2%	94.2%	93.4%	101.6%

Figure 55. Effect of different MIC endpoint definitions for conidial sequential antifungal testing (I→A) of NCPF 7097. Detection interval cutoff values are 20% (A), 30% (B), 40% (C), and 50% (D). Values in each well represent percent residual glucose. Grey boxes indicate residual glucose percentages that fall below the detection interval cutoff value. Red boxes indicate drug combinations for which FIC values would be determined.

A

81.7%	92.5%	90.3%	88.2%	77.3%	85.6%	83.4%	90.1%
91.4%	95.2%	81.4%	82.2%	73.0%	86.2%	79.6%	80.1%
88.3%	94.4%	79.4%	85.4%	73.5%	88.2%	79.3%	77.8%
89.9%	79.9%	80.2%	79.1%	72.5%	83.6%	75.2%	75.6%
79.1%	65.5%	72.3%	76.5%	73.7%	86.7%	73.5%	72.9%
75.4%	66.9%	56.7%	69.6%	91.9%	79.9%	72.3%	76.3%
77.7%	55.9%	58.2%	59.7%	73.8%	84.0%	73.0%	84.8%
72.9%	63.2%	62.0%	60.3%	62.7%	89.5%	81.3%	93.4%

B

81.7%	92.5%	90.3%	88.2%	77.3%	85.6%	83.4%	90.1%
91.4%	95.2%	81.4%	82.2%	73.0%	86.2%	79.6%	80.1%
88.3%	94.4%	79.4%	85.4%	73.5%	88.2%	79.3%	77.8%
89.9%	79.9%	80.2%	79.1%	72.5%	83.6%	75.2%	75.6%
79.1%	65.5%	72.3%	76.5%	73.7%	86.7%	73.5%	72.9%
75.4%	66.9%	56.7%	69.6%	91.9%	79.9%	72.3%	76.3%
77.7%	55.9%	58.2%	59.7%	73.8%	84.0%	73.0%	84.8%
72.9%	63.2%	62.0%	60.3%	62.7%	89.5%	81.3%	93.4%

C

81.7%	92.5%	90.3%	88.2%	77.3%	85.6%	83.4%	90.1%
91.4%	95.2%	81.4%	82.2%	73.0%	86.2%	79.6%	80.1%
88.3%	94.4%	79.4%	85.4%	73.5%	88.2%	79.3%	77.8%
89.9%	79.9%	80.2%	79.1%	72.5%	83.6%	75.2%	75.6%
79.1%	65.5%	72.3%	76.5%	73.7%	86.7%	73.5%	72.9%
75.4%	66.9%	56.7%	69.6%	91.9%	79.9%	72.3%	76.3%
77.7%	55.9%	58.2%	59.7%	73.8%	84.0%	73.0%	84.8%
72.9%	63.2%	62.0%	60.3%	62.7%	89.5%	81.3%	93.4%

D

81.7%	92.5%	90.3%	88.2%	77.3%	85.6%	83.4%	90.1%
91.4%	95.2%	81.4%	82.2%	73.0%	86.2%	79.6%	80.1%
88.3%	94.4%	79.4%	85.4%	73.5%	88.2%	79.3%	77.8%
89.9%	79.9%	80.2%	79.1%	72.5%	83.6%	75.2%	75.6%
79.1%	65.5%	72.3%	76.5%	73.7%	86.7%	73.5%	72.9%
75.4%	66.9%	56.7%	69.6%	91.9%	79.9%	72.3%	76.3%
77.7%	55.9%	58.2%	59.7%	73.8%	84.0%	73.0%	84.8%
72.9%	63.2%	62.0%	60.3%	62.7%	89.5%	81.3%	93.4%

Figure 56. Effect of different MIC endpoint definitions for conidial concurrent antifungal testing (V+A) of NCPF 7097. Detection interval cutoff values are 40% (A), 50% (B), 60% (C), and 90% (D). Values in each well represent percent residual glucose. Grey boxes indicate residual glucose percentages that fall below the detection interval cutoff value. Red boxes indicate drug combinations for which FIC values would be determined.

AMB→VRC on conidial inocula were primarily additivity and/or indifference. While AMB→VRC had a similar effect on hyphal inocula, AMB→ITC had a synergistic effect on the hyphal inocula of all three *A. fumigatus* strains. The most controversial sequential antifungal therapy is an azole (ITC or VRC) followed by AMB. Although some reports have described patients improving by this therapeutic sequence, the majority of reports indicate that patients die or are given a tertiary therapeutic regimen (218-225). Our results support the latter by consistently demonstrating ITC→AMB and VRC→AMB's antagonistic effects on both conidial and hyphal inocula. Like concurrent ITC+VRC testing, ITC→VRC and VRC→ITC had additive and/or indifferent effects on conidial and hyphal inocula.

The results of concurrent and sequential antifungal combination testing with the conidial and hyphal inocula of *A. fumigatus* strain NCPF 7100 (ITC-resistant) provided new insights as to why ITC→AMB and VRC→AMB antagonism occurs. As NCPF 7100 is resistant to ITC but sensitive to VRC, I was not surprised when VRC→AMB sequential testing resulted in an antagonistic effect. This result is consistent with the most popular hypothesis of why azole→AMB occurs, that the azole inhibits production of ergosterol, thus eliminating AMB's target. Based on this hypothesis, I predicted that resistance to ITC would eliminate ITC→AMB antagonism. Surprisingly, I also found that ITC→AMB resulted in antagonism. As the mechanism of ITC resistance has not been elucidated with *A. fumigatus* NCPF 7100, I conducted identical ITC→AMB sequential testing with the conidial and hyphal inocula of *A. fumigatus* AF-72, an ITC-resistant strain whose mechanism of ITC resistance has been attributed to a mutation in

the ITC target, P450 14α-demethylase (110). A mutation in the ITC target means that while the strain is resistant to ITC, ergosterol levels do not change in its presence. Again, ITC→AMB sequential testing resulted in antagonism (data not shown). I therefore concluded that ITC's effect on ergosterol production is not responsible for azole→AMB antagonism. Instead, the results support a less popular hypothesis of azole→AMB induced antagonism, which has thus far been investigated with only *C. albicans*; due to its lipophilicity, ITC binds to components on the fungal cell surface, blocking binding of AMB to ergosterol (177). However, additional studies are necessary to verify whether this is in fact occurring.

Based on the in vitro FIC index results of all of the *A. fumigatus* strains, I can make the following predictions of the clinical outcome using two-drug concurrent and sequential antifungal combination therapies of AMB, ITC, or VRC: AMB+ITC and AMB+VRC will not result in antagonism and may result in a efficacious effect than either therapy alone; ITC+VRC will not result in antagonism but does not provide a more efficacious outcome than either therapy used alone; ITC or VRC therapy as a secondary therapy after primary therapy of AMB will not result in antagonism; AMB should never be used as a secondary therapy when ITC or VRC has been used as a primary therapy; and ITC and VRC can be used interchangeably as primary and secondary therapies. However, these are only predictions and should not be used as a guide of antifungal therapy. Instead, these results should be confirmed in one or more IA animal models, and if synergy is observed, tested as a therapeutic regimen in a prospective clinical trial.

In conclusion, I have utilized in vitro checkerboard testing formats to determine the effects of concurrent and sequential antifungal combinations on the conidial and hyphal inocula of *A. fumigatus* clinical isolates and found that concurrent and sequential antifungal combinations can have differential effects on conidial and hyphal inocula. Antagonistic effects could be detected with both conidial and hyphal inocula while synergy was more readily detected with hyphal inocula. As hyphae are the morphological form of the fungus observed in vivo, I recommend that all future combination testing be performed with hyphal inocula.

CHAPTER 8

CONCLUSIONS TO DISSERTATION HYPOTHESES

Hypothesis 1

The yeast RSA was successfully modified to facilitate amphotericin B (AMB), itraconazole (ITC), and voriconazole (VRC) testing of *A. fumigatus*, *A. terreus*, and *A. flavus* isolates. The objective, 16 h mold RSA (mRSA) AMB, ITC, and VRC MICs were equal to or within a single, two-fold dilution of the subjective AMB, ITC, and VRC MICs determined by the National Committee for Clinical Laboratory Standards (NCCLS) M38-P and M38-A assays in 48 h and the Etest in 24 or 48 h. The mRSA therefore provides a more rapid and/or objective alternative to NCCLS M38-P, NCCLS M38-A, and Etest AMB, ITC, and VRC susceptibility testing of *A. fumigatus*, *A. terreus*, and *A. flavus*. Preliminary testing with *A. sydowii*, *Scedosporium angiospermum*, and *Fusarium oxysporum* isolates also suggests that the RSA may be used to determine the AMB and ITC susceptibilities of other filamentous fungi.

Hypothesis 2

The mRSA was successfully modified to determine the susceptibility of *A. fumigatus* hyphae to AMB, ITC, and VRC. The MICs of hyphal inocula were concentration-dependent, with optimal hyphal inocula prepared with conidial inocula of 0.11 optical density (OD_{530nm}) diluted 1:50, 1:100, or 1:200. AMB, ITC, and VRC MICs of hyphae were not significantly different than the AMB, ITC, and VRC MICs of

conidia. These data suggest that RSA testing can be performed with conidial or hyphal inocula. However, we recommend the use of conidial inocula as this facilitates generation of MICs in a shorter period of time.

Hypotheses 3 and 4

RSA FLC testing of FLC-resistant *Candida albicans* clinical isolates demonstrated that the yeast RSA was able to generate clinically relevant FLC MICs. Yeast RSA conditions were also determined to generate clinically relevant FLC MICs for *C. glabrata* clinical isolates.

Hypothesis 5

We were unable to use a RSA-based testing format for interpreting the effects of concurrent and sequential antifungal combinations on *A. fumigatus* conidial and hyphal inocula. We instead interpreted these effects by a visual assessment of growth. Based on this criterion, our interpretations of the in vitro effects of concurrent and sequential antifungal combinations on *A. fumigatus* closely matched the reported outcomes of invasive aspergillosis patients treated with concurrent and sequential antifungal therapies.

Hypothesis 6

Concurrent and sequential antifungal testing demonstrated that select drug combinations had differential effects on conidial and hyphal inocula. Specifically, AMB and ITC, ITC followed by AMB, or VRC followed by AMB had a greater synergistic

effect on hyphal, rather than conidial, inocula. In contrast, antagonistic effects could be detected with both conidial and hyphal inocula.

Hypothesis 7

Although RSA testing provided quantitative imput data for the ModLab program, interpretations of the interaction coefficients generated by the program did not consistently correspond to interaction effects determined by visual observation.

APPENDIX

ADDITIONAL DATA FROM CONCURRENT AND SEQUENTIAL ANTIFUNGAL COMBINATION TESTING

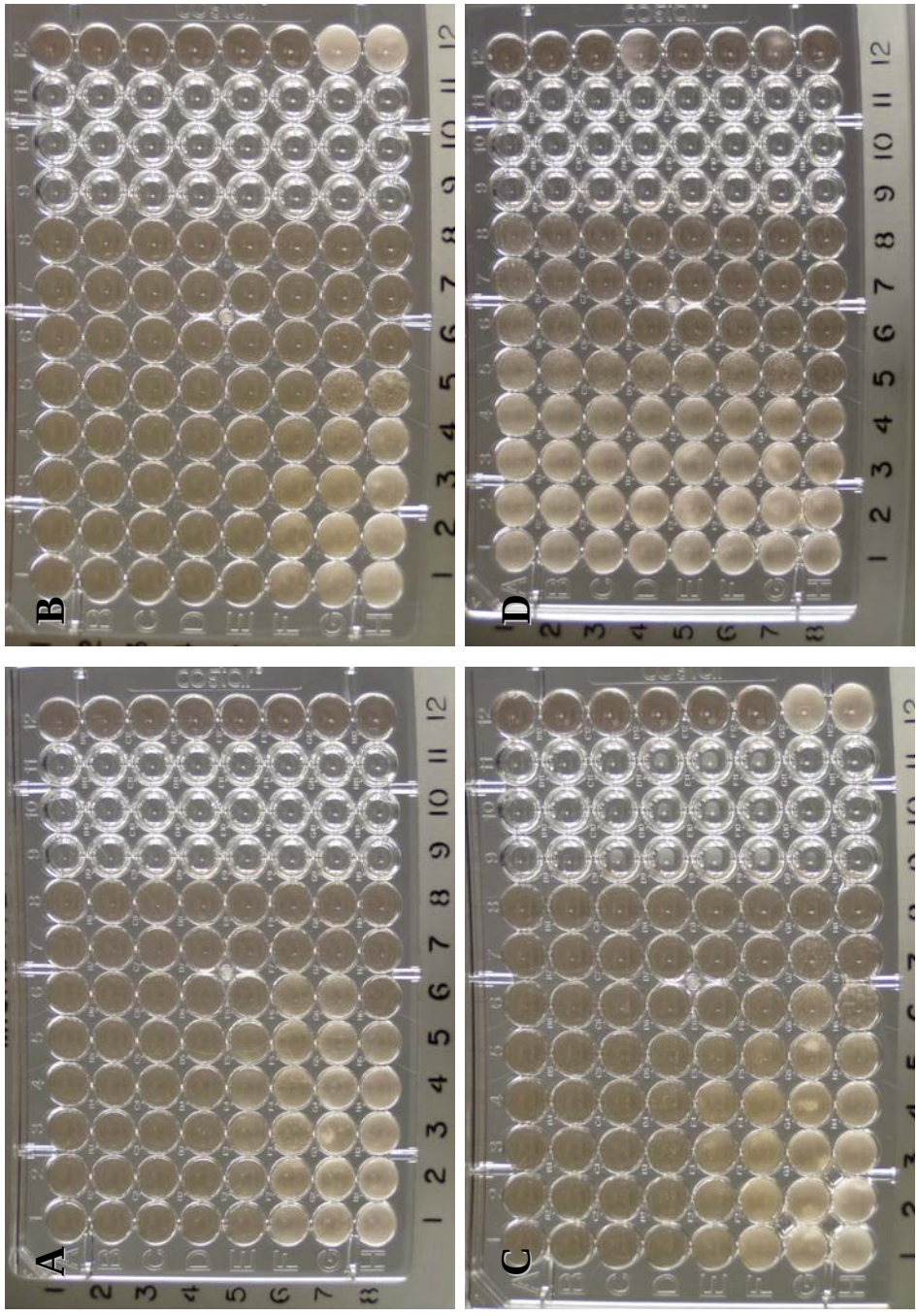


Figure 57. Visual growth of conidial inocula *A. fumigatus* strains NCPF 7101 (A), 7097 (B), 7098 (C), and 7100 (D) after 48 h concurrent AMB+ITC combination testing. AMB concentration increases on the horizontal axis from right to left. ITC concentration increases on the vertical axis from bottom to top.

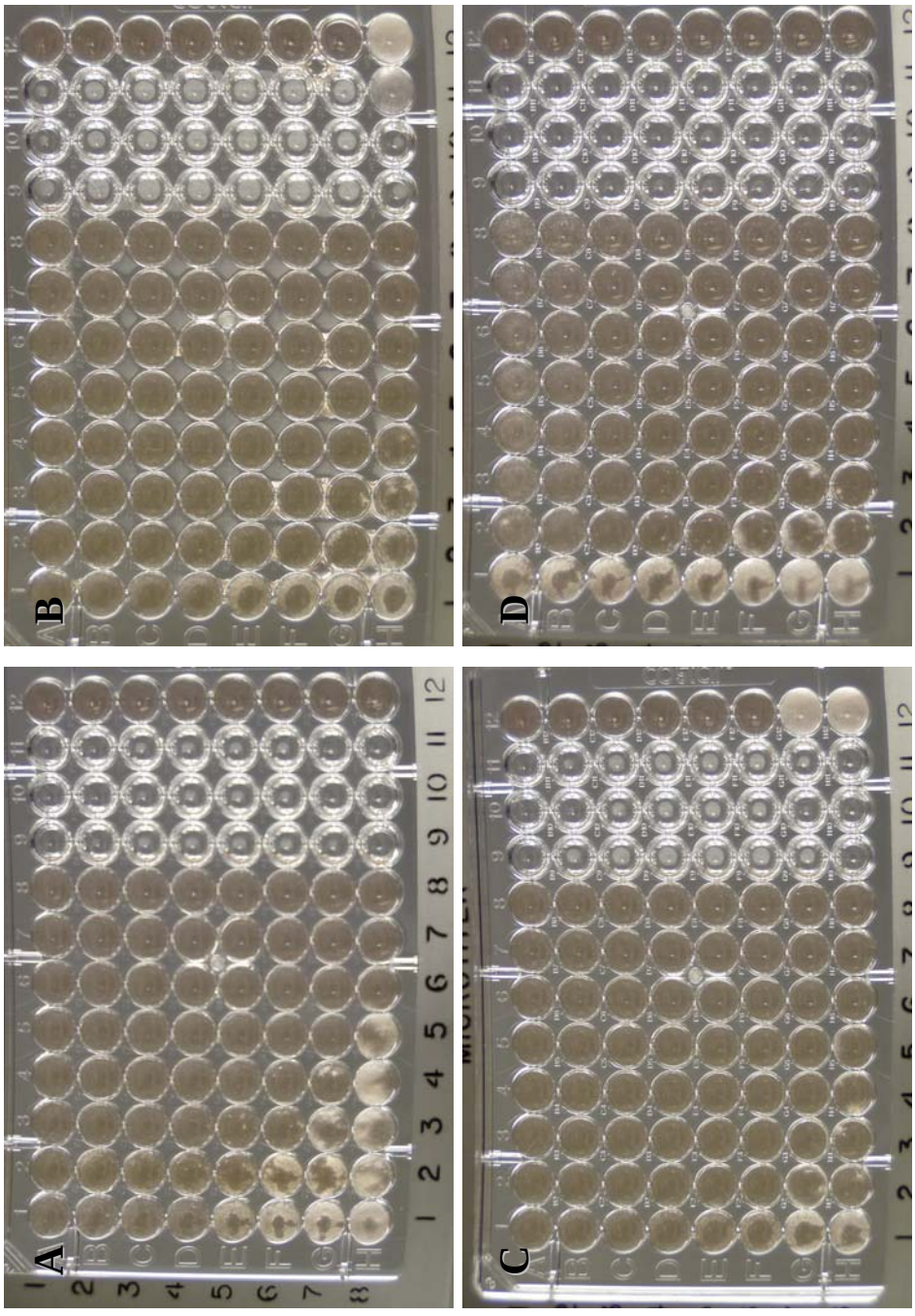


Figure 58. Visual growth of hyphal inocula *A. fumigatus* strains NCPF 7101 (A), 7097 (B), 7098 (C), and 7100 (D) after 48 h concurrent AMB+ITC combination testing. AMB concentration increases on the horizontal axis from right to left. ITC concentration increases on the vertical axis from bottom to top.

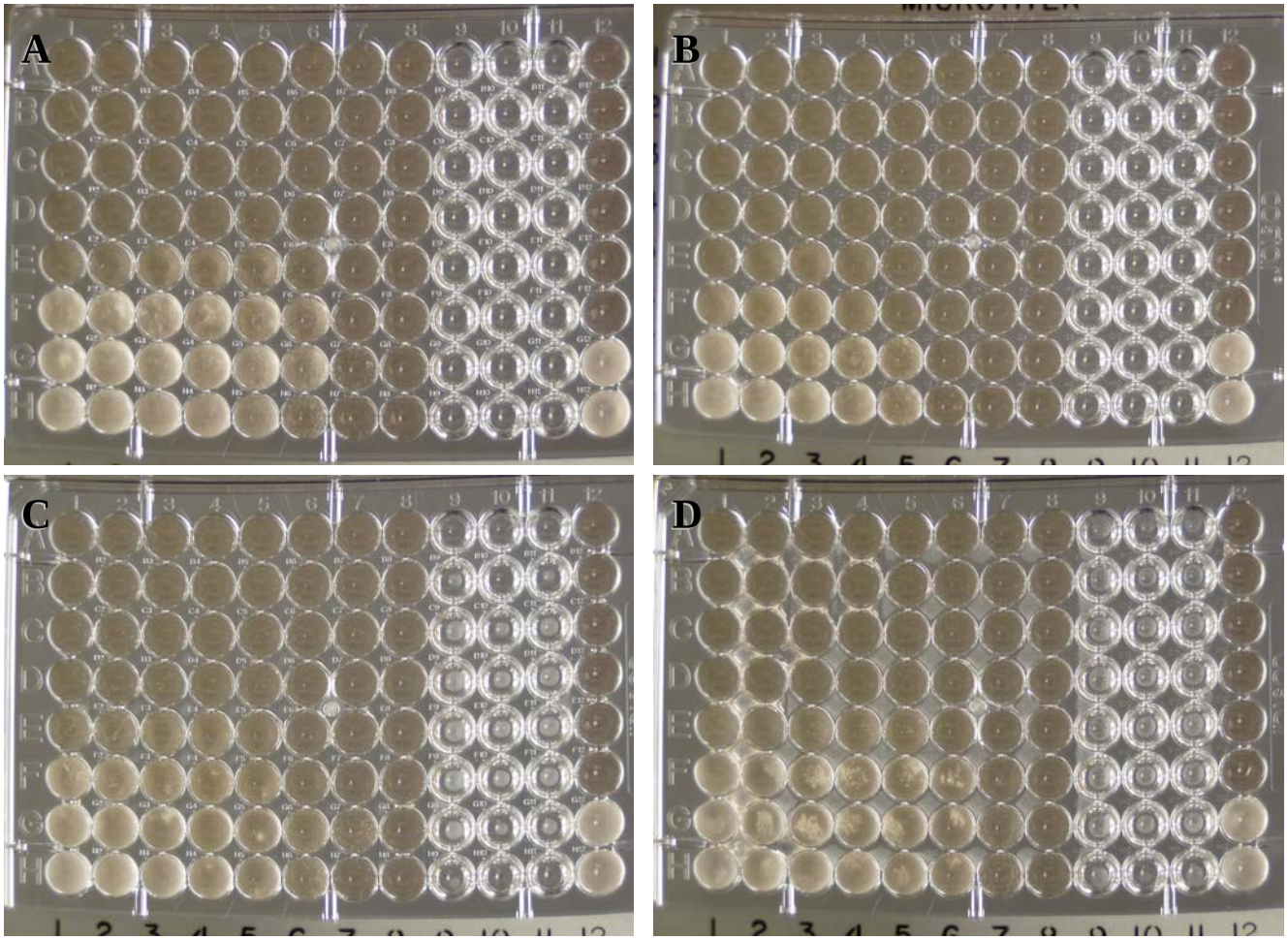


Figure 59. Visual growth of conidial inocula of *A. fumigatus* strains NCPF 7101 (A), 7097 (B), 7098 (C), and 7100 (D) after 48 h concurrent AMB+VRC combination testing. AMB concentration increases on the horizontal axis from right to left. VRC concentration increases on the vertical axis from bottom to top.

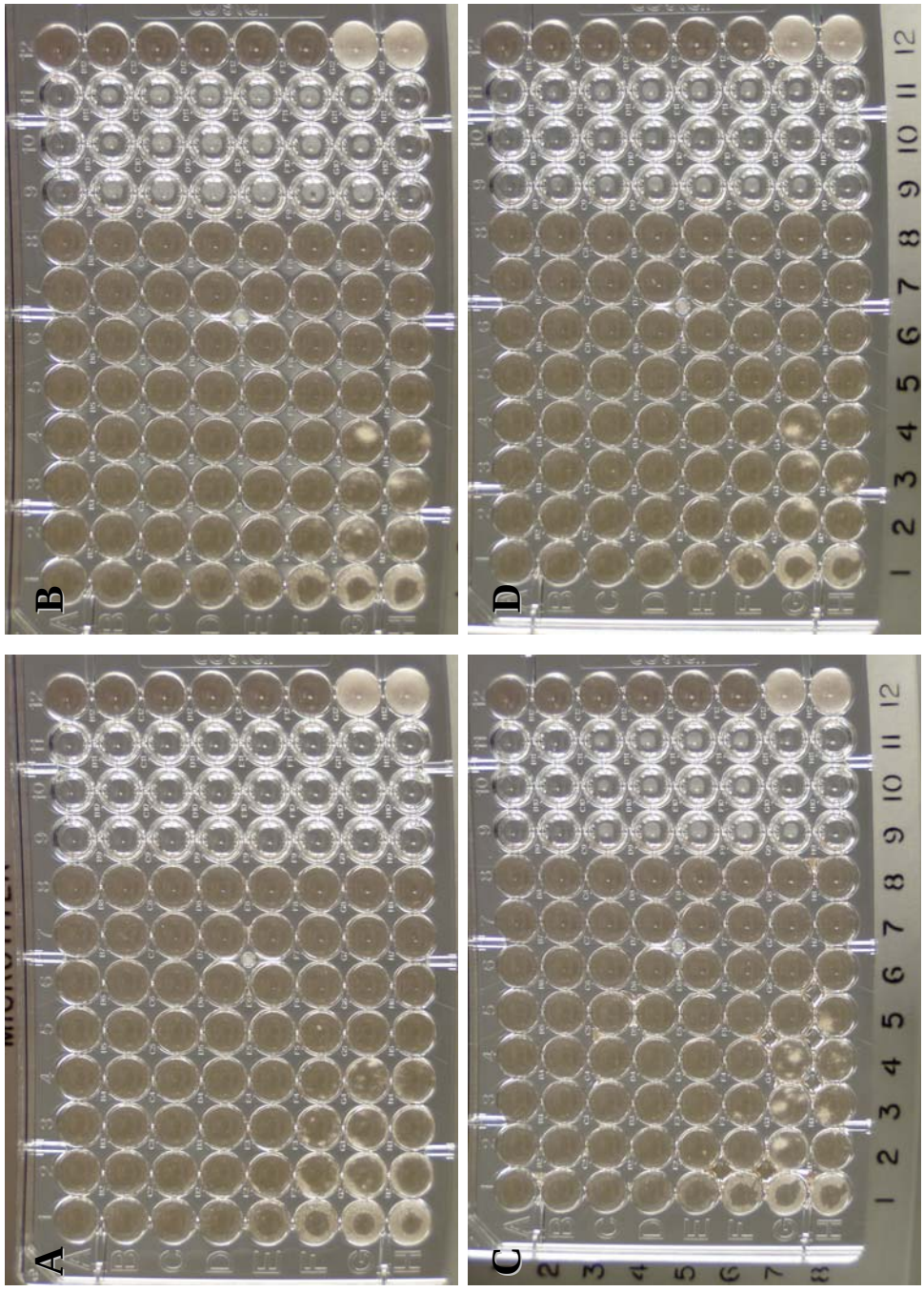


Figure 60. Visual growth of hyphal inocula of *A. fumigatus* strains NCPF 7101 (A), 7097 (B), 7098 (C), and 7100 (D) after 48 h concurrent AMB+VRC combination testing. AMB concentration increases on the horizontal axis from right to left. VRC concentration increases on the vertical axis from bottom to top.

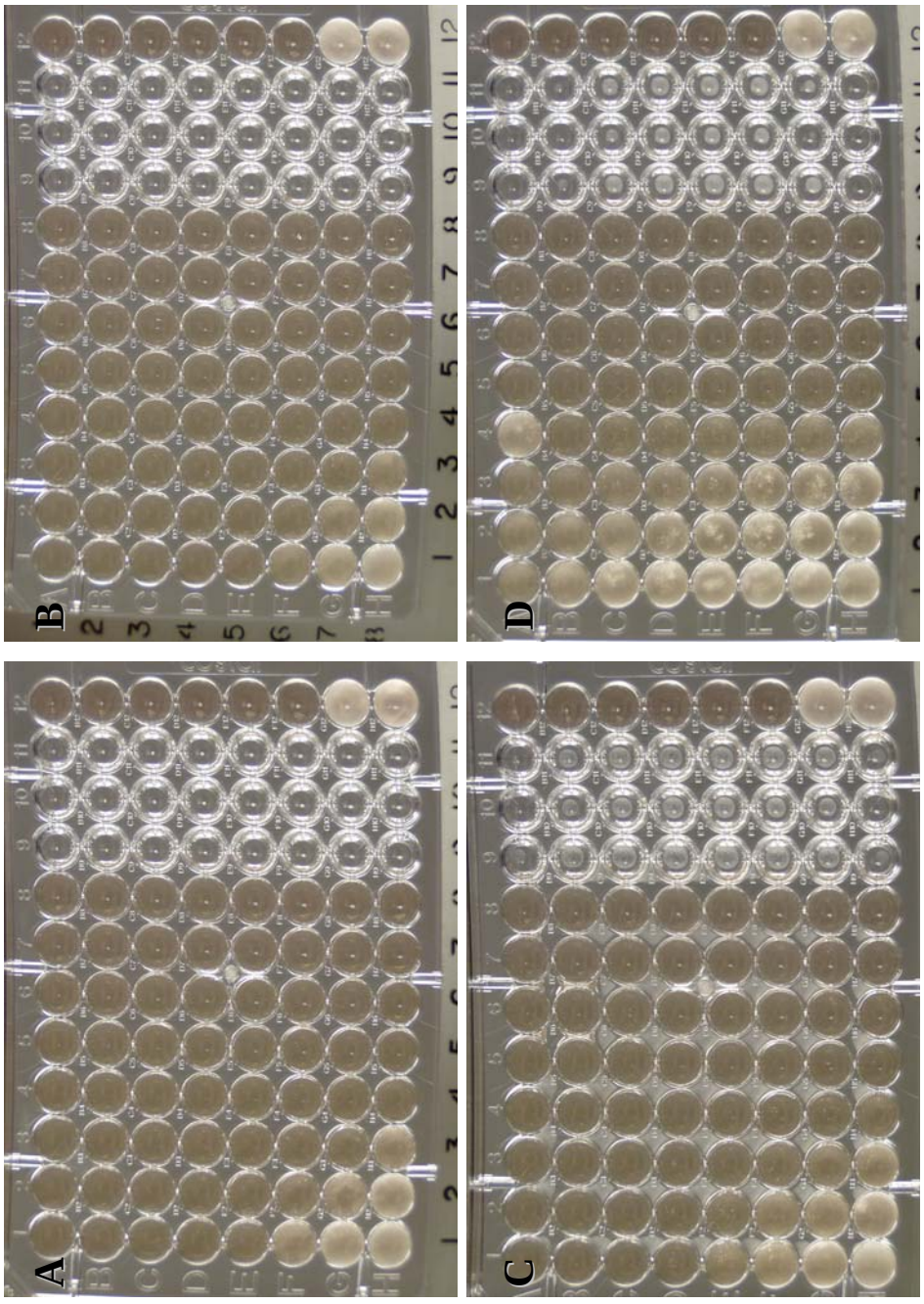


Figure 61. Visual growth of conidial inocula of *A. fumigatus* strains NCPF 7101 (A), 7097 (B), 7098 (C), and 7100 (D) after 48 h concurrent ITC+VRC combination testing. VRC concentration increases on the horizontal axis from right to left. ITC concentration increases on the vertical axis from bottom to top.

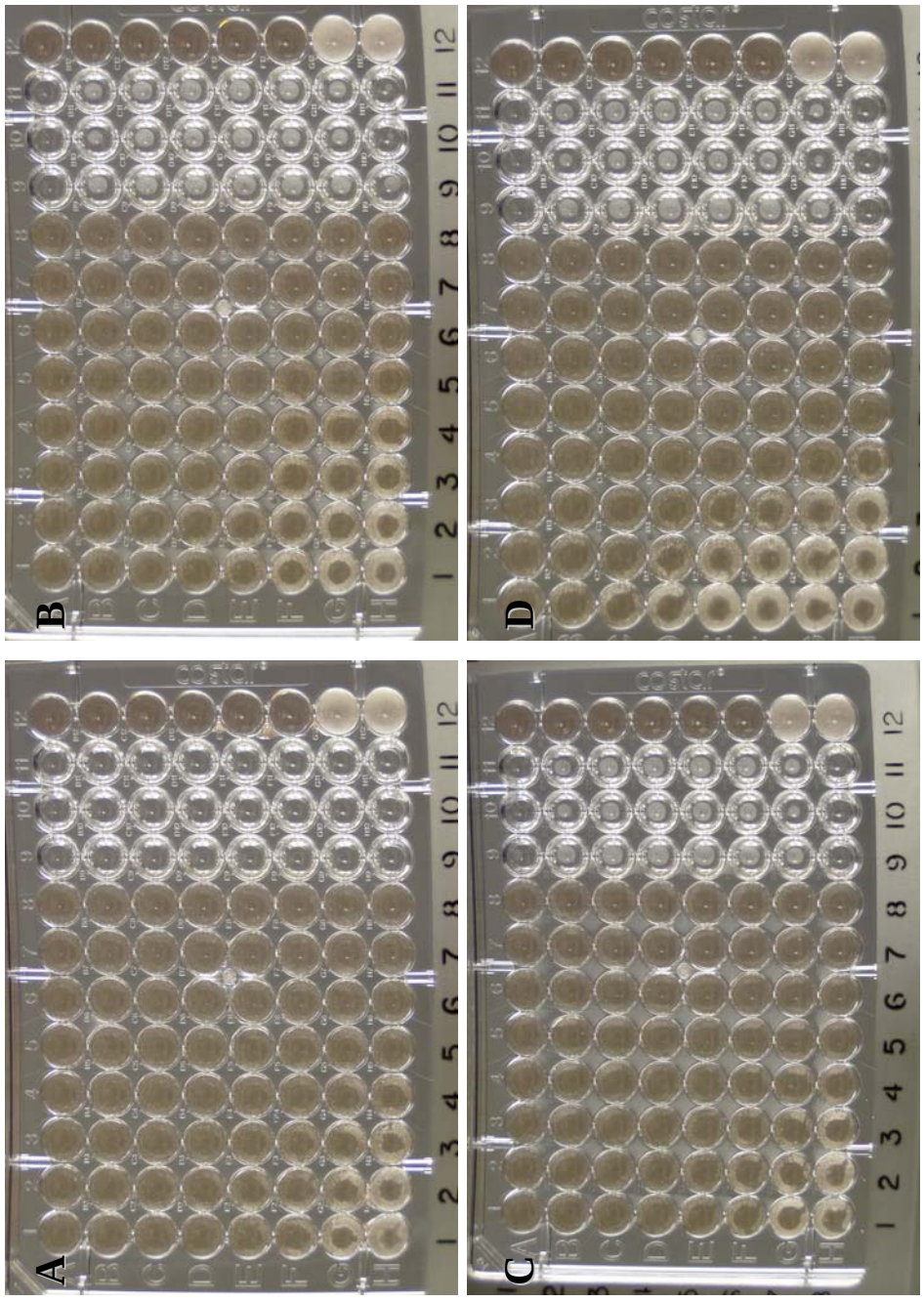


Figure 62. Visual growth of hyphal inocula of *A. fumigatus* strains NCPF 7101 (A), 7097 (B), 7098 (C), and 7100 (D) after 48 h concurrent ITC+VRC combination testing. VRC concentration increases on the horizontal axis from right to left. ITC concentration increases on the vertical axis from bottom to top.

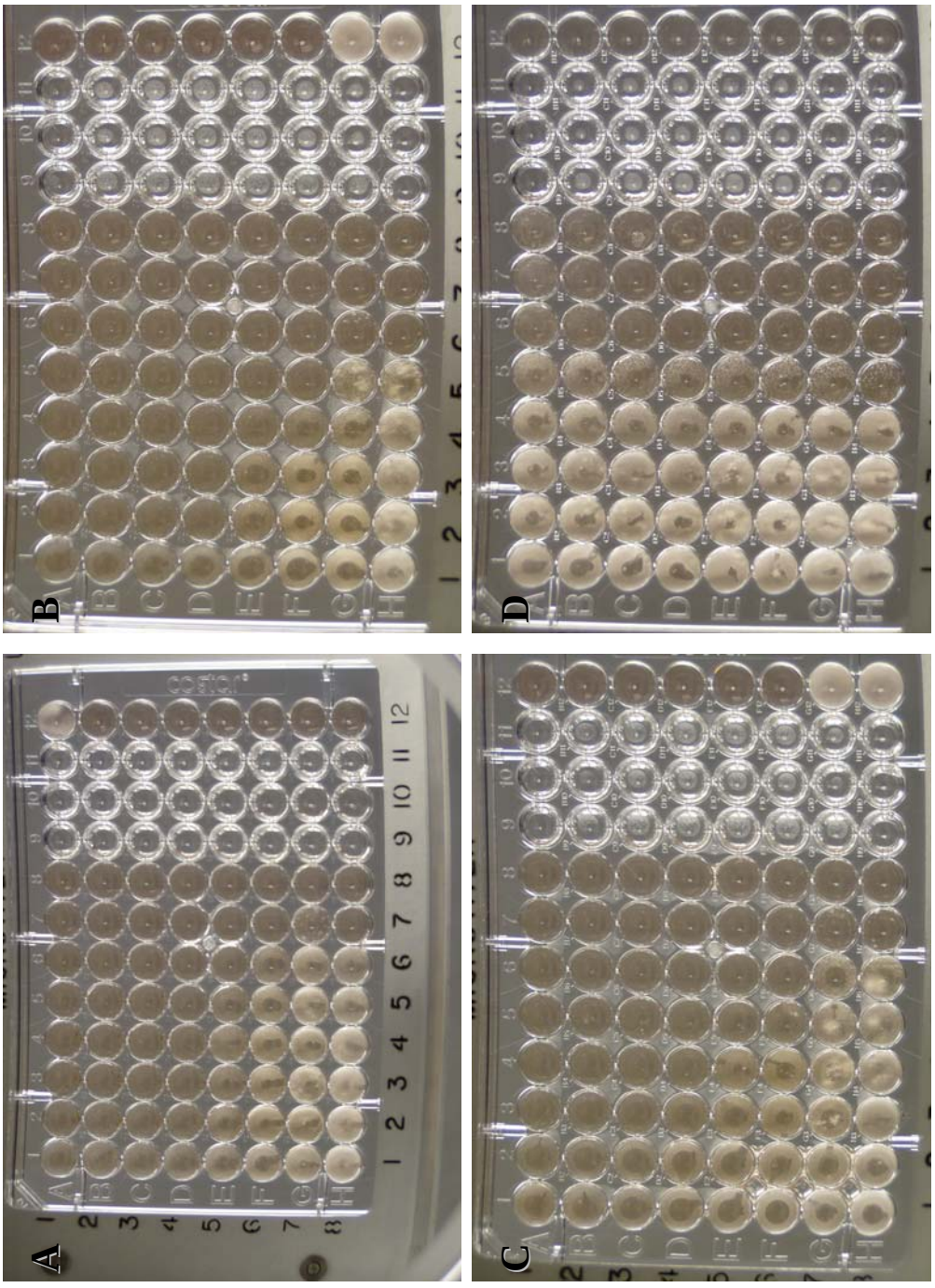


Figure 63. Visual growth of conidial inocula of *A. fumigatus* strains NCPF 7101 (A), 7097 (B), 7098 (C), and 7100 (D) after 48 h sequential AMB→ITC combination testing. AMB concentration increases on the horizontal axis from right to left. ITC concentration increases on the vertical axis from bottom to top.

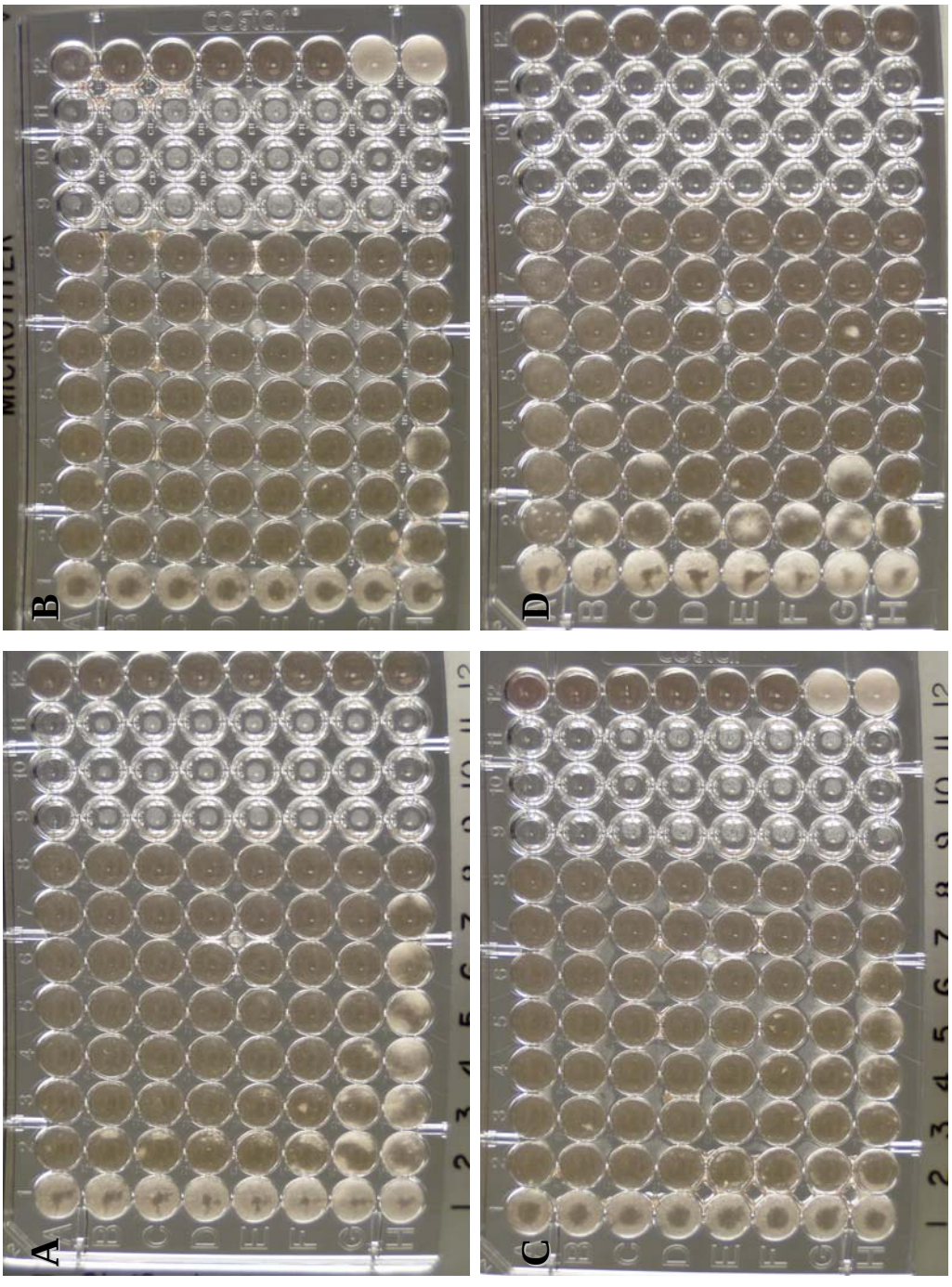


Figure 64. Visual growth of hyphal inocula of *A. fumigatus* strains NCPF 7101 (A), 7097 (B), 7098 (C), and 7100 (D) after 48 h sequential AMB→ITC combination testing. AMB concentration increases on the horizontal axis from right to left. ITC concentration increases on the vertical axis from bottom to top.

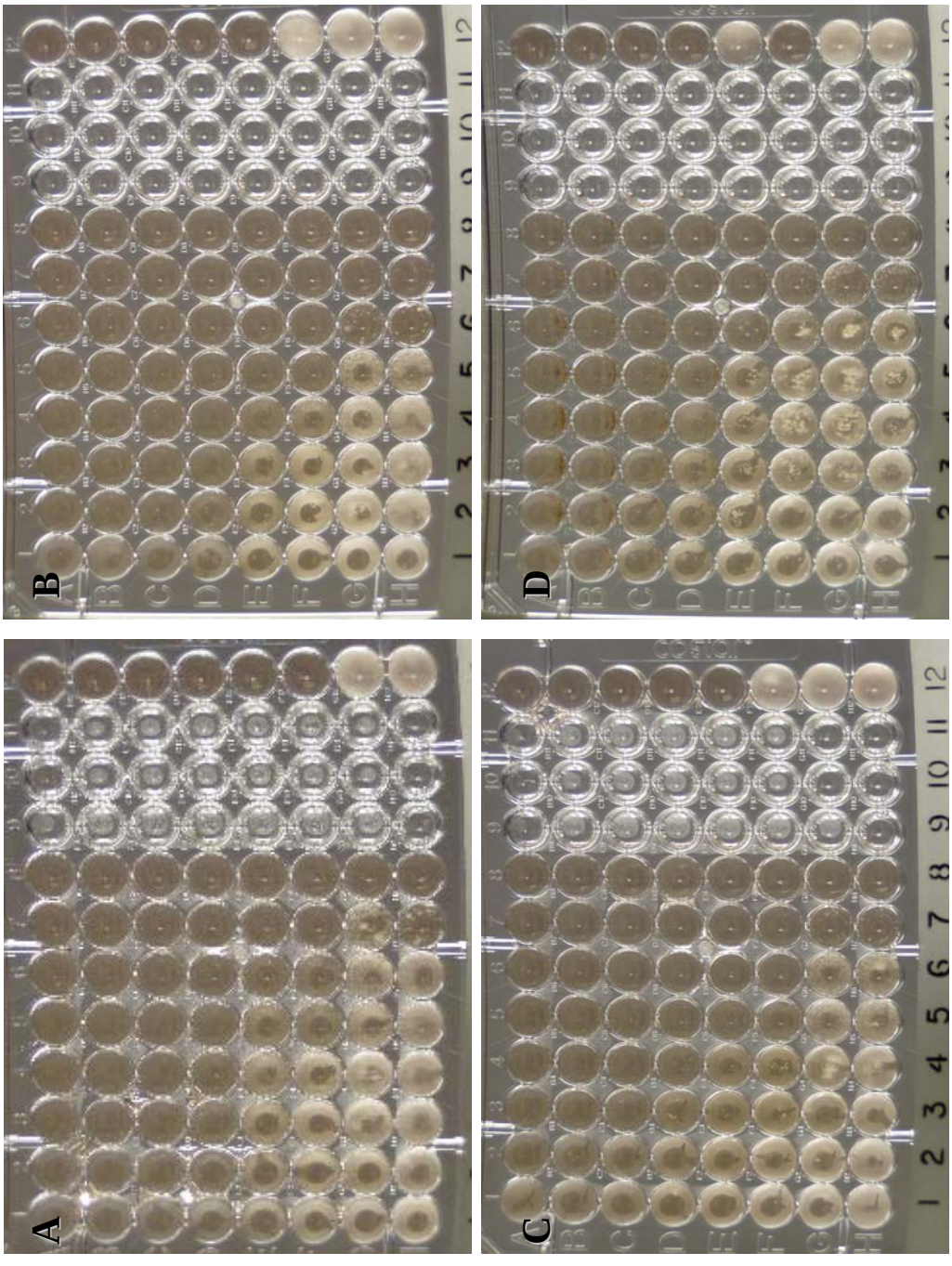


Figure 65. Visual growth of conidial inocula of *A. fumigatus* strains NCPF 7101 (A), 7097 (B), 7098 (C), and 7100 (D) after 48 h sequential AMB→VRC combination testing. AMB concentration increases on the horizontal axis from right to left. VRC concentration increases on the vertical axis from bottom to top.

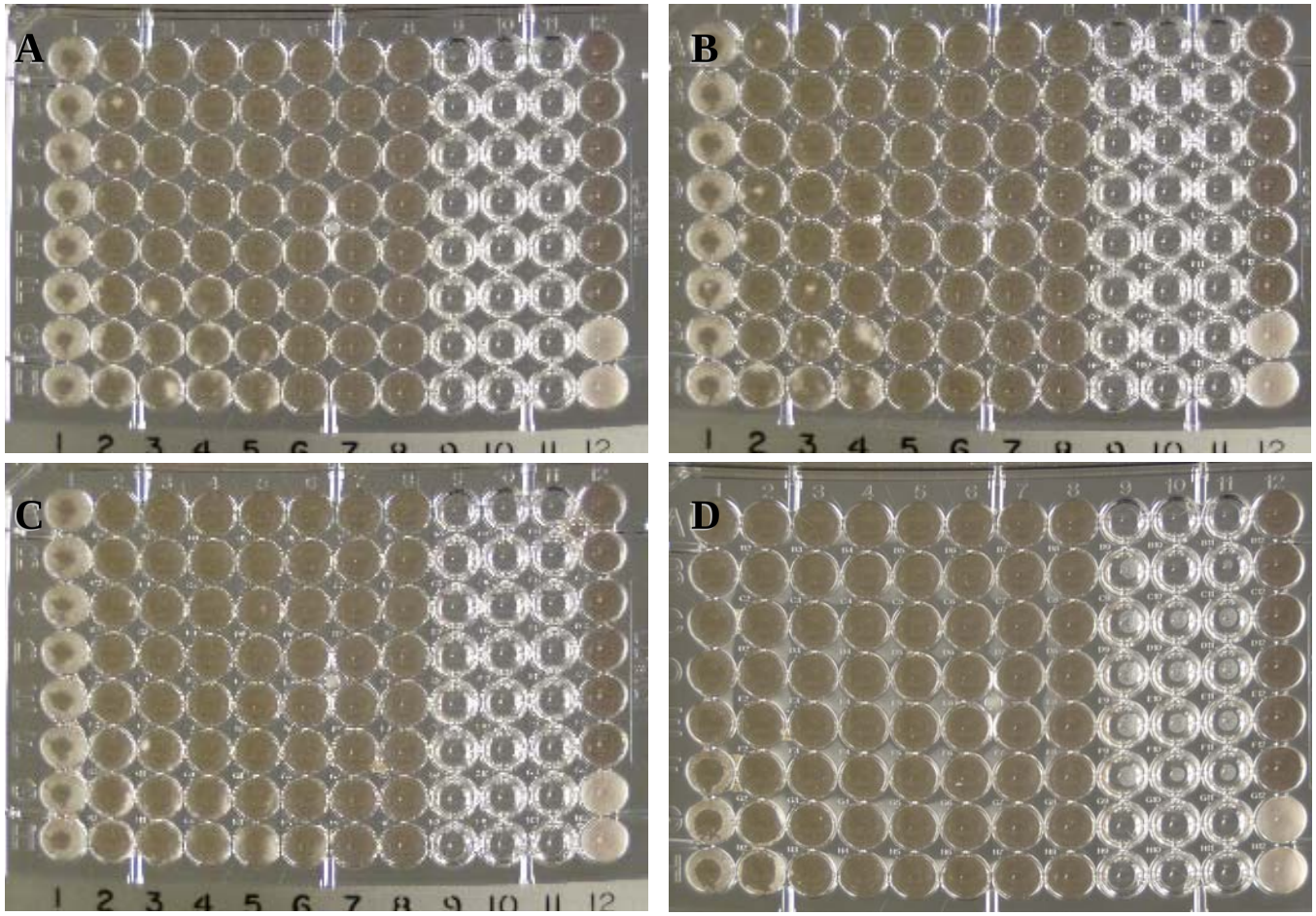


Figure 66. Visual growth of hyphal inocula of *A. fumigatus* strains NCPF 7101 (A), 7097 (B), 7098 (C), and 7100 (D) after 48 h sequential AMB→VRC combination testing. AMB concentration increases on the horizontal axis from right to left. VRC concentration increases on the vertical axis from bottom to top.

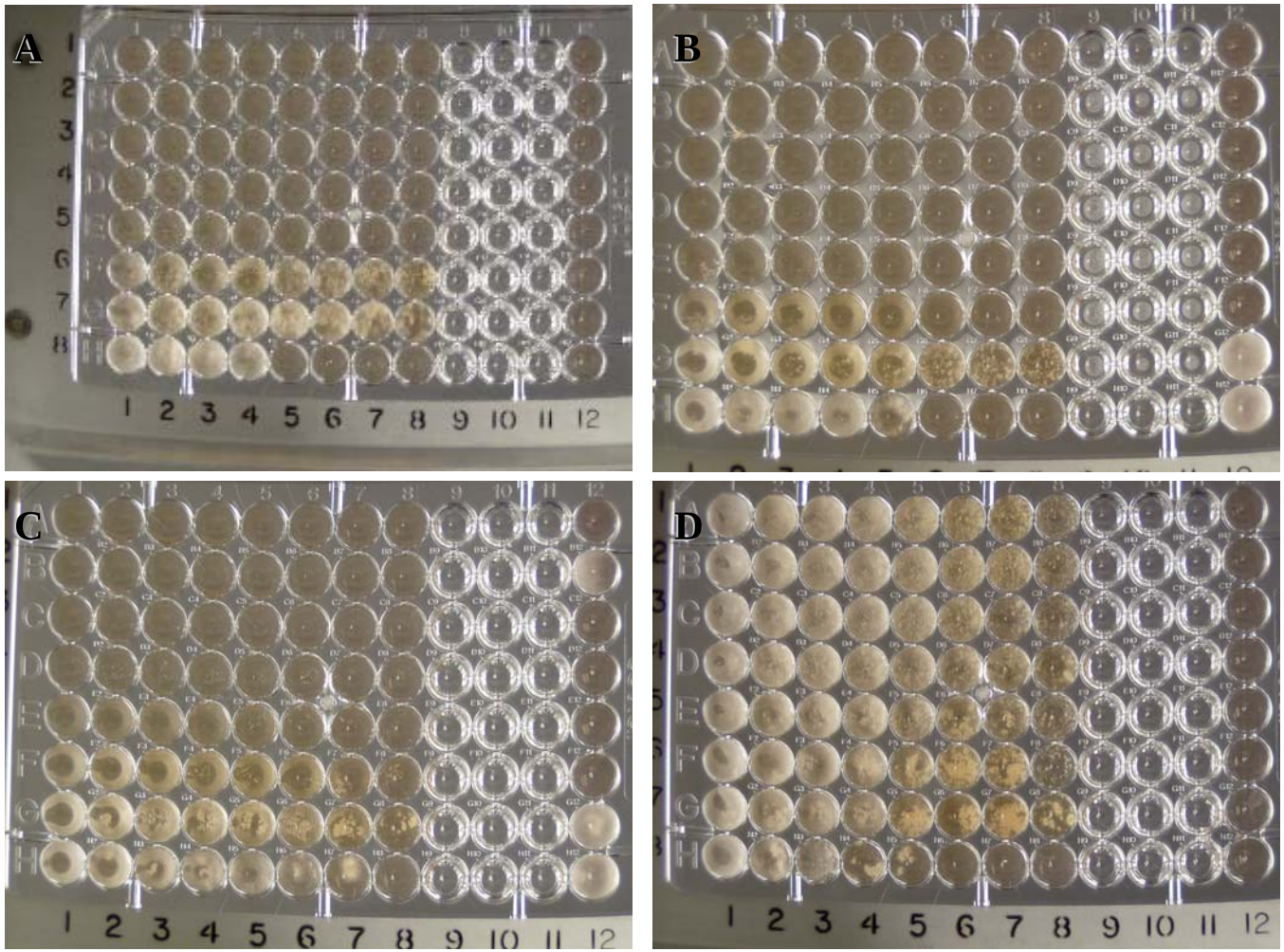


Figure 67. Visual growth of conidial inocula of *A. fumigatus* strains NCPF 7101 (A), 7097 (B), 7098 (C), and 7100 (D) after 48 h sequential ITC→AMB combination testing. AMB concentration increases on the horizontal axis from right to left. ITC concentration increases on the vertical axis from bottom to top.

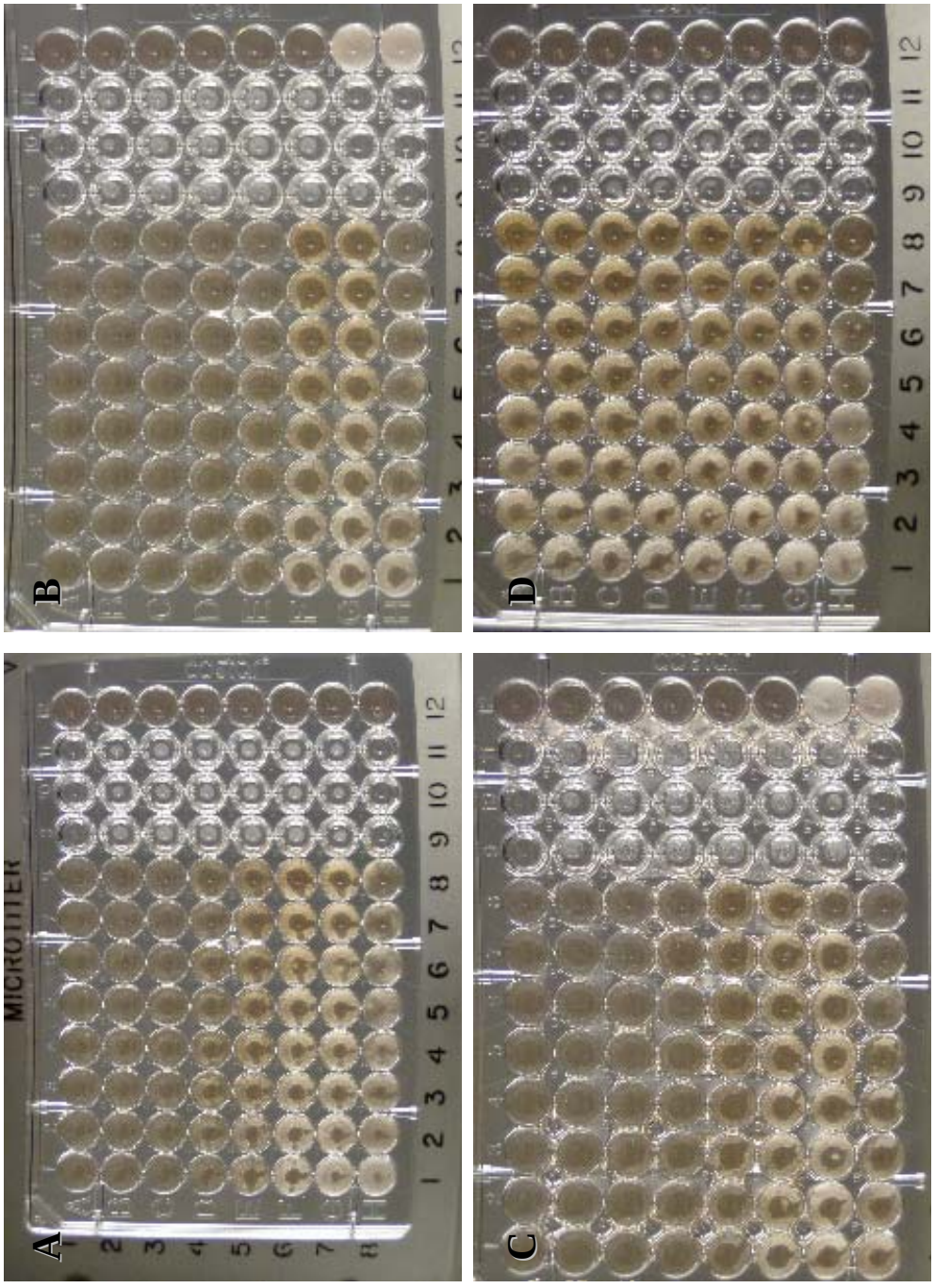


Figure 68. Visual growth of hyphal inocula of *A. fumigatus* strains NCPF 7101 (A), 7097 (B), 7098 (C), and 7100 (D) after 48 h sequential ITC→AMB combination testing. AMB concentration increases on the horizontal axis from right to left. ITC concentration increases on the vertical axis from bottom to top.

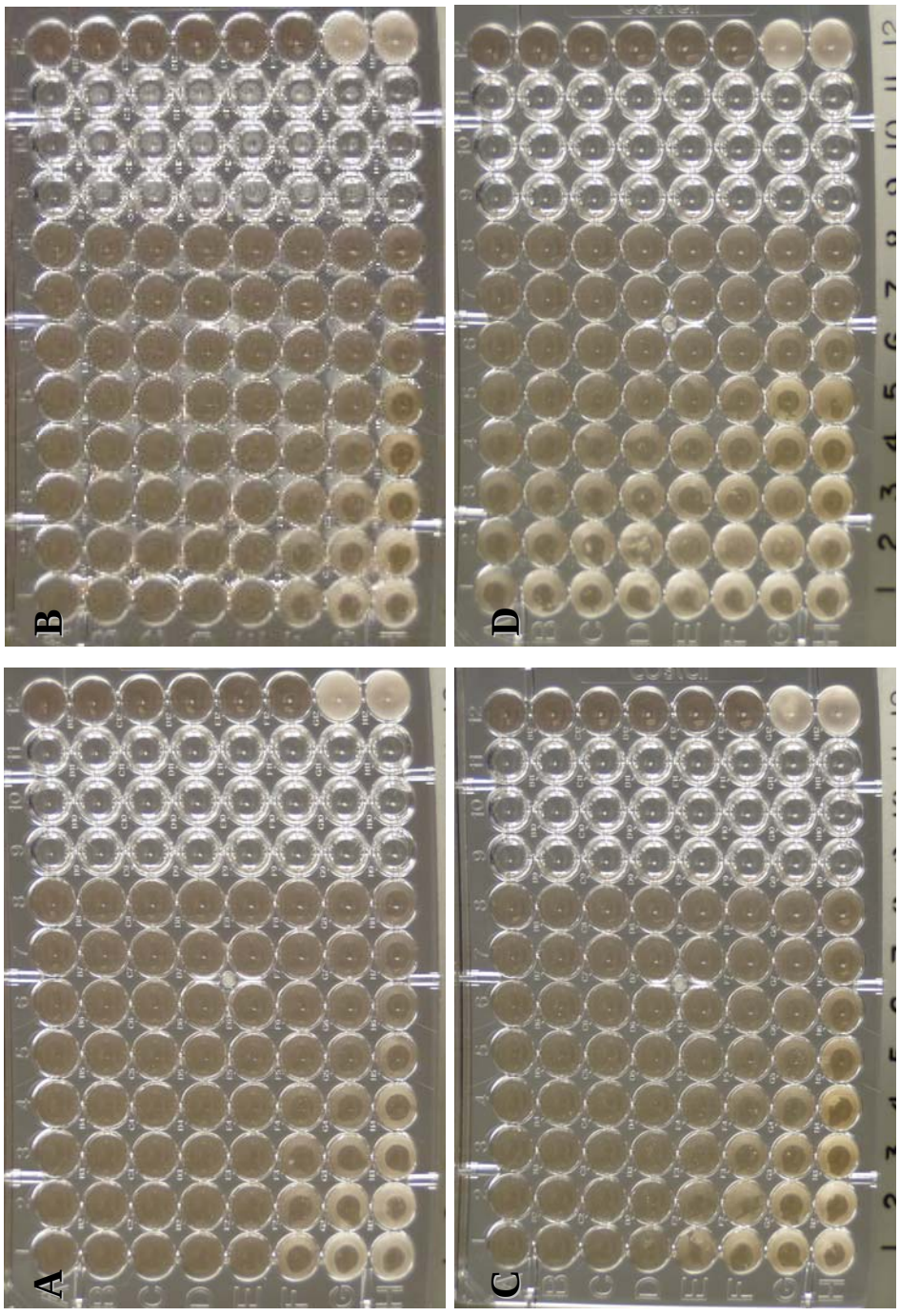


Figure 69. Visual growth of conidial inocula of *A. fumigatus* strains NCPF 7101 (A), 7097 (B), 7098 (C), and 7100 (D) after 48 h sequential ITC→VRC combination testing. VRC concentration increases on the horizontal axis from right to left. ITC concentration increases on the vertical axis from bottom to top.

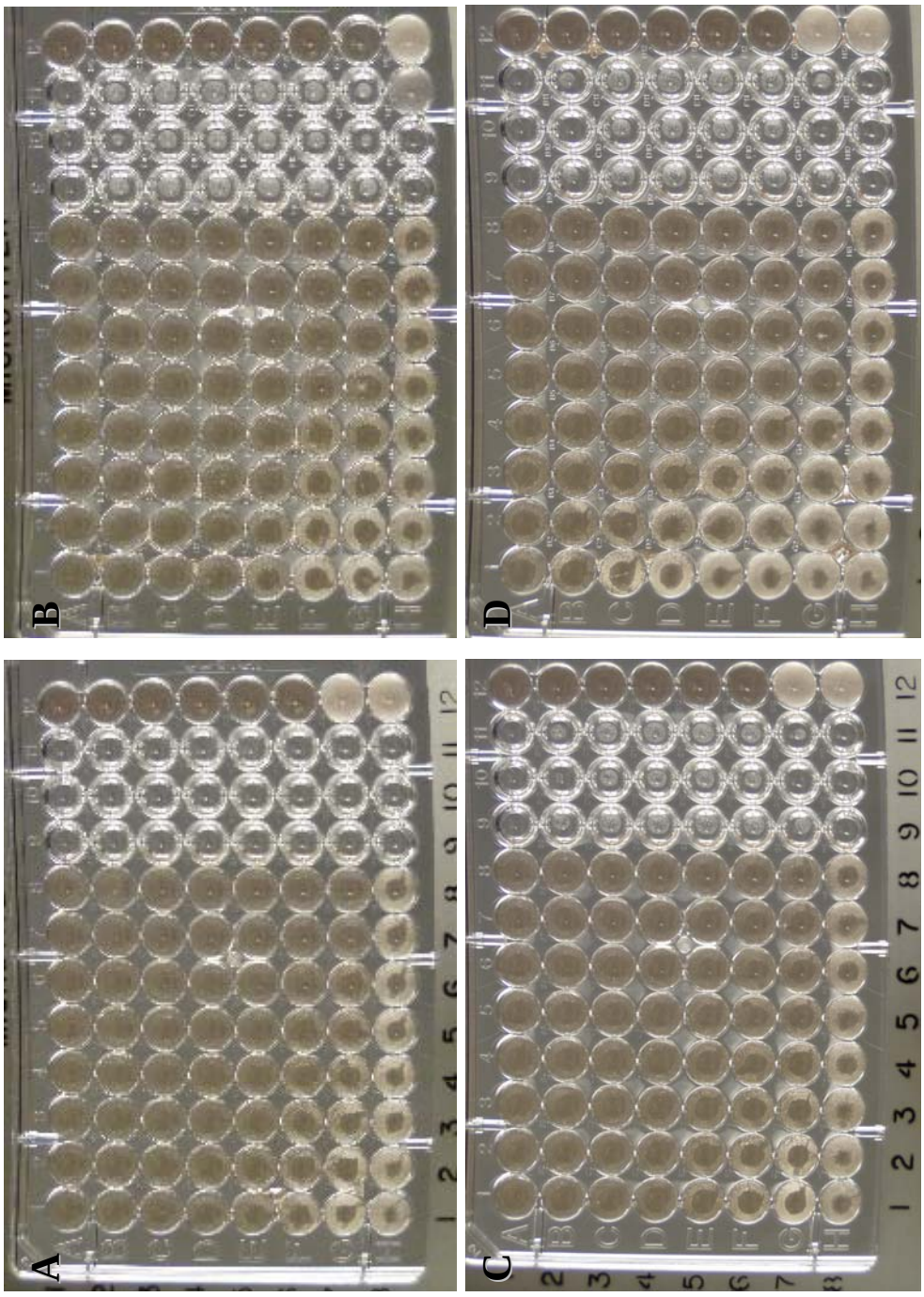


Figure 70. Visual growth of hyphal inocula of *A. fumigatus* strains NCPF 7101 (A), 7097 (B), 7098 (C), and 7100 (D) after 48 h sequential ITC→VRC combination testing. VRC concentration increases on the horizontal axis from right to left. ITC concentration increases on the vertical axis from bottom to top.

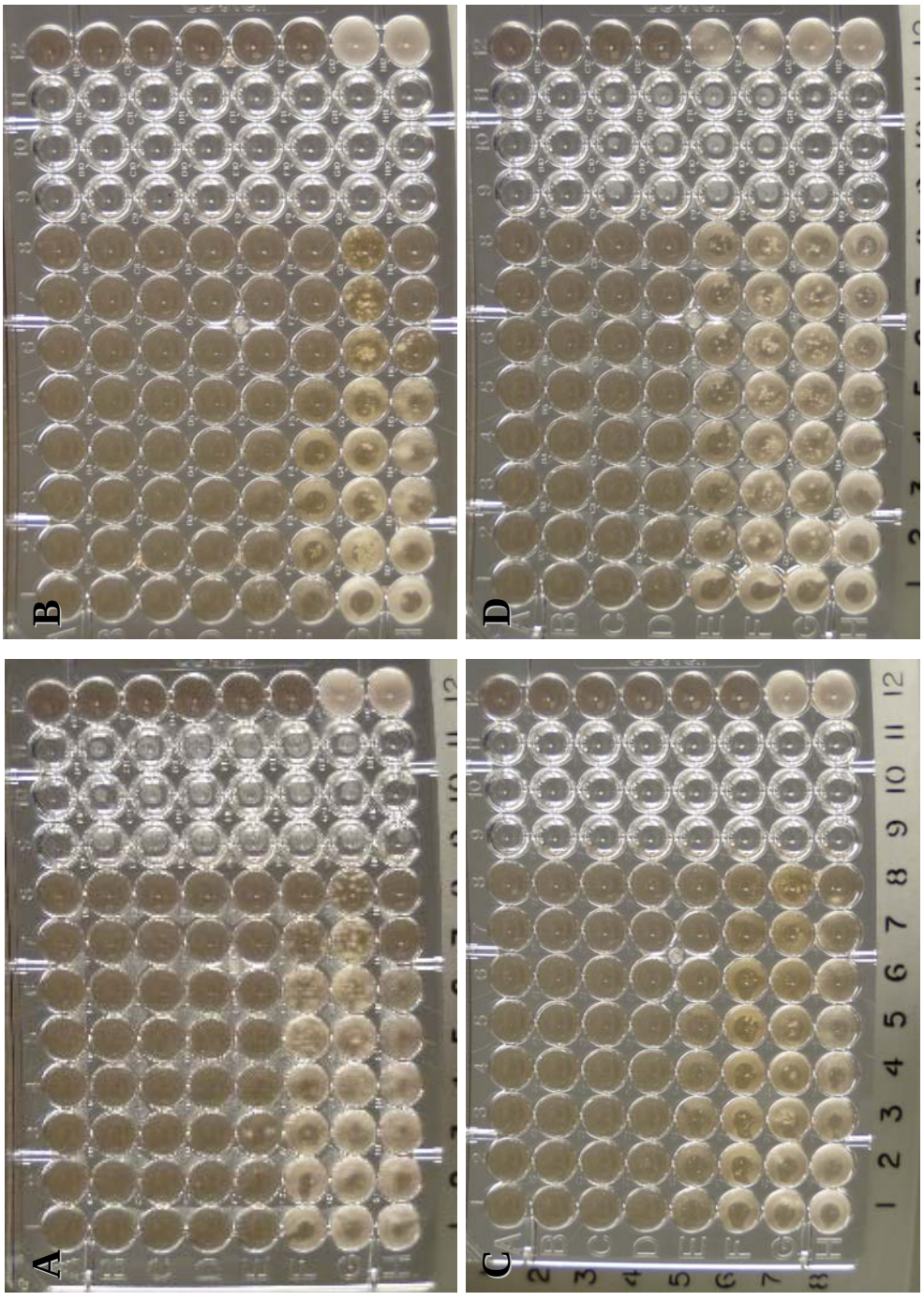


Figure 71. Visual growth of conidial inocula of *A. fumigatus* strains NCPF 7101 (A), 7097 (B), 7098 (C), and 7100 (D) after 48 h sequential VRC→AMB combination testing. AMB concentration increases on the horizontal axis from right to left. VRC concentration increases on the vertical axis from bottom to top.

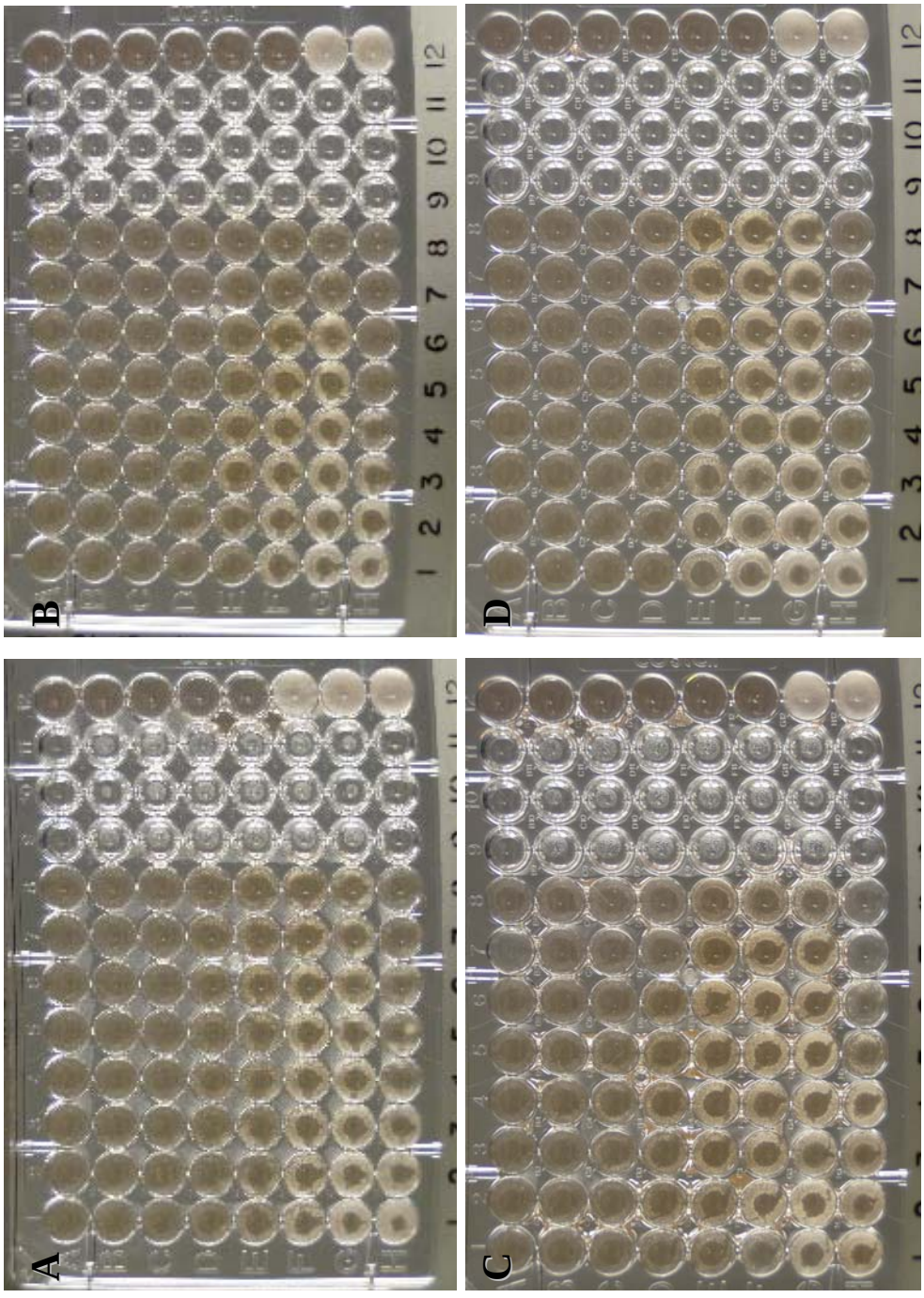


Figure 72. Visual growth of hyphal inocula of *A. fumigatus* strains NCPF 7101 (A), 7097 (B), 7098 (C), and 7100 (D) after 48 h sequential VRC→AMB combination testing. AMB concentration increases on the horizontal axis from right to left. VRC concentration increases on the vertical axis from bottom to top.

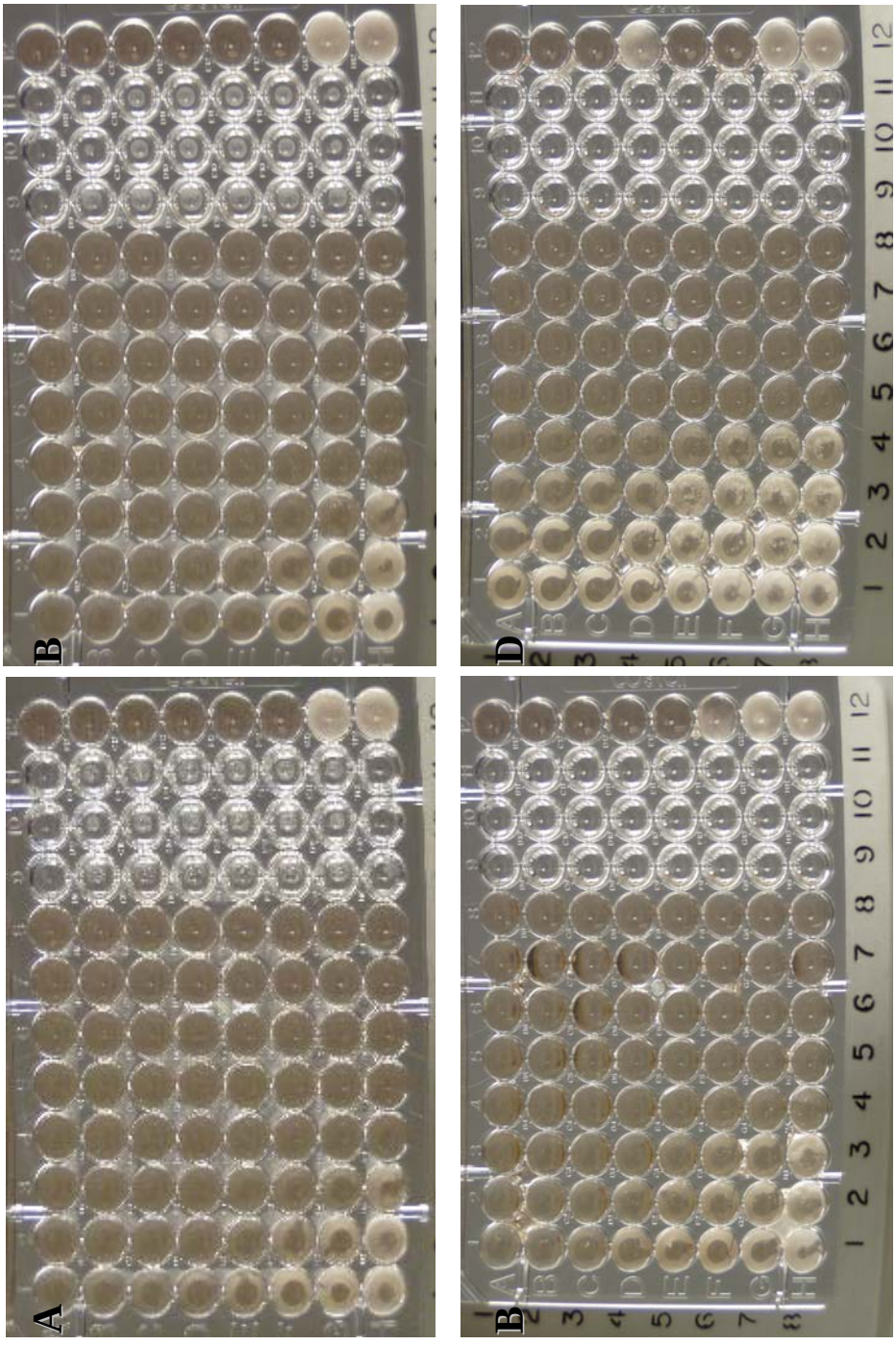


Figure 73. Visual growth of conidial inocula of *A. fumigatus* strains NCPF 7101 (A), 7097 (B), 7098 (C), and 7100 (D) after 48 h sequential VRC→ITC combination testing. VRC concentration increases on the horizontal axis from right to left. ITC concentration increases on the vertical axis from bottom to top.

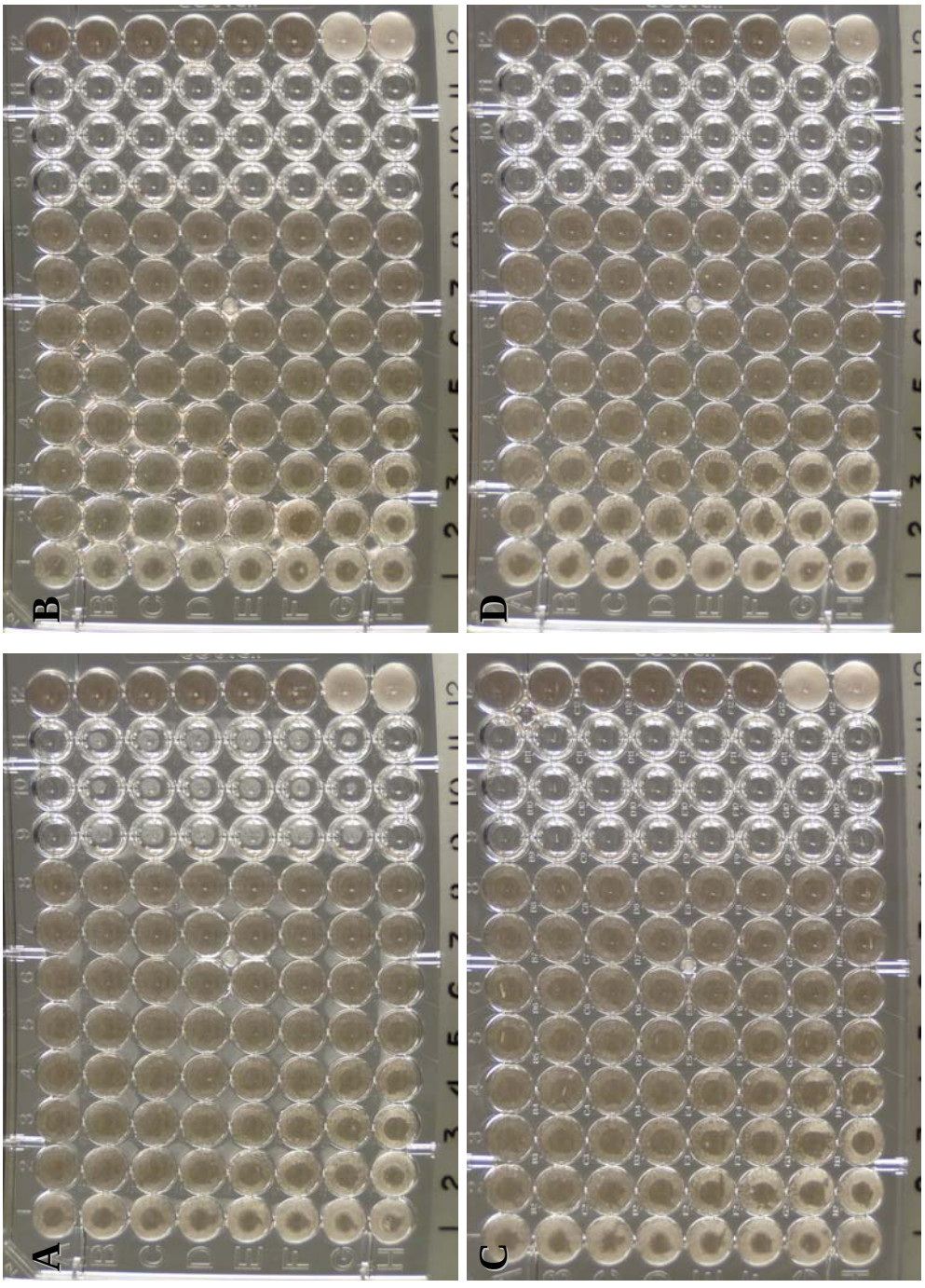


Figure 74. Visual growth of hyphal inocula of *A. fumigatus* strains NCPF 7101 (A), 7097 (B), 7098 (C), and 7100 (D) after 48 h sequential VRC→ITC combination testing. VRC concentration increases on the horizontal axis from right to left. ITC concentration increases on the vertical axis from bottom to top.

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