Determination of Fungicidal Activities against Yeasts and Molds: Lessons Learned from Bactericidal Testing and the Need for Standardization

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INTRODUCTION

In vitro antimicrobial susceptibility testing (AST) is performed routinely in most clinical microbiology laboratories to assess the relative susceptibility of microbial pathogens to selected therapeutic agents as an aid in optimizing the treatment of infectious diseases. AST has been performed with antibacterial agents for decades, and recently these methods have been adapted, standardized, and validated for testing antifungal agents against yeasts (National Committee for Clinical Laboratory Standards [NCCLS] M27-A2) (54) and filamentous fungi (NCCLS M38-A) (55). AST methods include agar and broth dilution and agar diffusion (disk and E test) tests, all of which measure the inhibitory activity of the tested antimicrobial agents. In the vast majority of clinical settings, an assessment of inhibitory activity is sufficient since the role of the antimicrobial agent is to minimize the spread of the infecting organism from the site of infection while the host defenses, coupled with other therapeutic interventions (e.g., removal of foreign body, surgical resection, or drainage), ultimately achieve eradication of the organism and clinical cure of the infection. Although it may be useful in the treatment of infection for an antimicrobial agent to have fungicidal activity, it is not essential to measure such activity in order to optimize treatment in most clinical situations (27, 52, 53, 61).

In certain unique clinical settings, however, the ability of the agent administered to kill the pathogen outright may be quite important (52, 53, 61). These situations invariably involve infection of a site not easily accessed by host defenses and/or of
a structure with essential anatomic or physiologic function such as the heart (endocarditis), central nervous system (CNS) (meningitis), or bone (osteomyelitis) (12-14, 22, 23, 38, 41, 48, 51, 68, 86, 87). Likewise, infections in immunosuppressed hosts, especially those who are neutropenic, are often thought to require microbiidal therapy (74).

Most of the clinical data supporting the need for microbioidal therapy have focused on bacterial infections. However, given the fact that most serious fungal infections occur in profoundly immunosuppressed individuals, it is generally assumed that a “cidual” regimen would be preferable in that setting as well (2, 9, 25, 39, 47, 57, 66, 73).

Proof of the cidual nature of an antifungal agent in vitro is tedious, complex, and fraught with error (52, 53, 61). Although several methods for assessing in vitro bactericidal activity have been standardized (NCCLS M26-A and M21-A [52, 53]), the clinical relevance of these determinations is questionable and the tests are performed infrequently in most laboratories (52, 53, 61).

The steady increase in the number of immunocompromised individuals has resulted in an ever-growing number of serious fungal infections due to both common (e.g., Candida albicans and Aspergillus fumigatus) and uncommon (e.g., Candida krusei, Trichosporon spp., and Fusarium spp.) fungal pathogens (47, 56, 66, 70, 71, 73). The introduction of several new systemically active antifungal agents with increased spectrum and potency and, in some instances, novel mechanisms of action offers new hope for improved therapeutic outcomes and has resulted in a perceived need to assess the fungicidal activity of these agents (2, 3, 6, 9, 11, 20, 21, 35, 45, 47, 57, 66, 70, 71). Given that most invasive fungal infections take place in patients with severely compromised or nonexistent host defenses, this is not an unreasonable expectation. In view of this clinical concern, we considered that it would be useful to first briefly review what is known about the issues and problems in assessing bactericidal activity and the clinical utility of such measurements and then discuss the issue of how one defines fungicidal activity, in vivo and in vitro, and how feasible it might be to determine the fungicidal activity of organism-drug combinations for purposes of both drug development and clinical care.

METHODOLOGY FOR DETERMINING THE MICROBICIDAL ACTIVITY OF ANTIBACTERIAL AGENTS

Bactericidal activity can be determined by a number of in vitro methods including the time-kill kinetic studies (single agent, fixed concentration; single agent, multiples of MIC; and combinations, synergy versus antagonism), MBC determinations and the serum bactericidal test (SBT) (bactericidal titer, bactericidal rate) (52, 53). Such methods for assessing bactericidal activity are considered “special” AST methods because they are not routinely applied to all microorganisms but, rather, are applied in selected situations (endocarditis, meningitis, or osteomyelitis) where bactericidal activity has been shown to be advantageous (52, 53). An additional need for these methods arises in the preclinical stage of drug development as new classes of antimicrobial agents are introduced and an assessment of their bactericidal activity by established methods is indicated. Several nonstandardized methods, including vital dyes and measurements of biomass (ATP), have been used to assess bactericidal activity but are not addressed in this review.

Standardized Methods

The National Committee for Clinical Laboratory Standards (NCCLS) has developed guidelines for performing MBC and time-kill studies (M26-A) (53) and for SBT determination (M21-A) (52). It is intended that these guidelines provide a uniform methodology for bactericidal testing that is practical to perform in most clinical microbiology laboratories. The development of such methodologic guidelines does not mean that such testing is necessarily clinically relevant; however, it does allow such testing to be performed in a standardized fashion in order to assess the clinical relevance of the results (52, 53).

Time-kill kinetic studies. Determination of the killing of a bacterial isolate over time by one or more antimicrobial agents under carefully controlled conditions is known as the time-kill method (53). This is a broth-based method where the rate of killing of a fixed inoculum (usually 5 × 10^6 CFU/ml) is determined by sampling control (organism, no drug) and antimicrobial agent-containing tubes or flasks at intervals (usually 0, 4, 8, 10 to 12, and 24 h of incubation) and determining the survivor colony count (CFU per milliliter) by spreading each sample onto an agar plate. The kill curves are constructed by plotting the CFU per milliliter surviving at each time point in the presence and absence of the antimicrobial agent. When used to assess a single antimicrobial agent, kill curves are constructed by testing several multiples of the MIC. When used to determine synergy or antagonism between two (or more) antimicrobial agents, kill curves are constructed for a fixed concentration of each agent alone and in combination and are compared to that of the drug-free control at each time point. The definition of bactericidal activity usually requires ≥99.9% killing of the final (starting) inoculum and is determined by noting the presence or absence of a 3-log₁₀-unit decrease in the CFU per milliliter (53). When testing combinations of two or more agents, synergy is defined as a ≥2-log₁₀-unit decrease in the CFU per milliliter between the combination and its most active constituent after 24 h of incubation, with the less active component being tested at an ineffective concentration. In some instances, the actual rate of killing, seen between 4 and 8 h of incubation, may be more important than the concentration of antimicrobial at which 99.9% killing of the final inoculum occurs (7, 48, 61, 81). The time-kill method has been used widely for the evaluation of new antimicrobial agents and allows the determination of whether an agent produces concentration-dependent killing (where the extent of killing increases with increased drug concentrations) or time-dependent killing (where the killing continues only as long as the concentrations are in excess of the MIC) (53). Time-kill studies are used infrequently to guide therapy in an individual patient; however, interest in such studies is sustained, given the fact that (i) killing curves show good correlation with cure in experimental models of endocarditis (22, 61), (ii) time-kill studies are useful in determining tolerance to the lethal activity of antibacterial agents (12, 28, 68, 82) (see below), and (iii) the time-kill method is useful for determining synergy or antagonism between two (or more) antimicrobial agents (4, 5, 53). This

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method has been standardized and described in detail in NCCLS document M26-A (53).

**MBC determination.** The MBC test, also known as the minimal lethal concentration (MLC), is the most common estimation of bactericidal activity and is defined as the lowest concentration of antimicrobial agent needed to kill 99.9% of the initial inoculum after incubation for 24 h under a standardized set of conditions (53). The MBC test is a dilution test that most commonly employs twofold dilutions of the test agent in broth but may be performed as an agar dilution test as well. Broth dilution MBC tests first require performance of the standardized broth dilution technique for MIC tests, followed by sampling of dilutions having no visible growth after 24 h incubation and spreading of the sample onto the surface of an agar plate to determine the number of surviving CFU per milliliter (53, 58). The bactericidal end point (MBC) has been somewhat arbitrarily defined as the lowest concentration at which 99.9% of the final inoculum is killed (53, 58). The MBC may be determined using a macrodilution method (1 to 2 ml per tube) or a microdilution method (0.1 to 0.2 ml in each well); however, the microdilution method appears to offer greater reproducibility and is used more commonly in clinical microbiology laboratories (27, 59, 61, 79). Determination of the MBC is strongly influenced by methodologic variables, and for that reason it has been very difficult to assess the clinical utility of MBC determination (27, 59, 61, 79). A standardized method for performing both macro- and microdilution MBC determinations using broth medium is described in NCCLS document M26-A (53).

**Serum bactericidal test.** The SBT integrates both pharmacokinetic and pharmacodynamic properties in a single set of determinations that examines the ability of the patient’s serum, drawn at various times during the dosing interval, usually at the beginning and end of the administered antimicrobial agent, to kill the infecting organism (7, 22, 69, 74, 76, 77, 86, 87). This can be done using dilution methodology to determine the maximum dilution or “titer” of the patient’s serum which demonstrates 99.9% killing of the final inoculum (serum bactericidal titer). Alternatively, a time-kill method may be used to determine the rate of killing of the infecting organism (change in CFU per milliliter per hour of exposure) produced by exposure to a 1:2 dilution of the patient’s serum obtained at the beginning (peak) and end (trough) of the dosing interval (7). As with time-kill and MBC determinations, the SBT is affected by methodological variables and has the additional complications of the need to properly collect timed serum specimens (7, 22, 52, 69, 76, 86, 87). Interpretation of test results is also problematic, and the clinical role of the SBT is controversial at best (52, 69, 76, 86, 87). The number of clinical situations in which the test may be useful is limited (e.g., endocarditis, osteomyelitis, and septic arthritis); however, it has been valuable in the research setting and in the evaluation of new antimicrobial agents. A standardized method for the SBT is provided in NCCLS document M21-A (52).

**Problems with Bactericidal Determinations**

Determination of the in vitro bactericidal activity of antimicrobial agents has a theoretical appeal, has been shown to be useful as a research tool, and may have a limited role clinically. Unfortunately, numerous biological and technical factors exist that can interfere with the performance of the various assays and make the interpretation of the results quite difficult. These factors are discussed in this section (53).

**Biological factors.** (i) **Persisters.** The bactericidal activity of certain antimicrobial agents, notably β-lactams, is directly related to the rate of bacterial growth (10, 26, 30, 49, 53, 58, 61, 79, 81, 88). The lower the rate of growth, the slower the bactericidal effect. In an in vitro test, some of the bacterial cells may be dormant or replicating slowly and thus are not killed by the otherwise bactericidal agent. These “persisters” usually constitute <0.1% of the final inoculum but will be apparent as growth on agar medium during the course of time-kill or MBC testing (8, 26, 49, 53, 79, 88). Notably, when retested these persisters appear just as susceptible as the parent strain and do not constitute a resistant subpopulation (8). Although a similar situation may exist in vivo, the clinical importance of persisters observed during the course of bactericidal testing is unclear.

(ii) **Paradoxical effect.** The paradoxical effect is manifested when the proportion of surviving cells increases significantly as the concentration of the antimicrobial agent increases beyond the MBC (15, 46, 53). Seen most commonly with the β-lactam agents (15, 53) but also seen with aminoglycosides (46), this effect is thought to occur in vitro when the growth of the organism is slowed to the extent that the lethal action of the drug is circumvented. The clinical relevance of the paradoxical effect is unclear (15, 53).

(iii) **Tolerance.** Tolerance occurs when an organism is inhibited but not killed by an agent that normally is considered bactericidal (12, 22, 28, 51, 53, 68, 75, 78, 82). Again, this is described most commonly for β-lactam agents and usually with gram-positive cocci (12, 22, 51, 68, 78, 82). Among the four putative mechanisms that may produce tolerance, two of these, persisters and the paradoxical effect, are described above. Among the remaining two mechanisms, phenotypic tolerance may be observed with any organism under certain growth conditions such that the organism is killed rapidly during the first few hours of drug exposure but its growth plateaus out at a higher survival rate (>0.1%) by 24 h of exposure (53, 82). The remaining mechanism is that in which an organism has a genetic trait, such as a defective autolytic system, that results in a greatly reduced rate of killing as determined by time-kill studies (82). Irrespective of the mechanism of tolerance, all organisms exhibiting this trait are characterized by unusually high drug MBCs relative to the MIC. Tolerance has been defined by an MBC-MIC ratio of ≥32 after 24 h of incubation (82). Time-kill studies are necessary to distinguish the specific mechanism involved for a given bacterial strain. Again, the clinical relevance of tolerance is controversial; however, limited data do suggest that tolerance may be an important consideration in the treatment of *Staphylococcus aureus* endocarditis (12, 22, 68, 82).

(iv) **Phenotypic resistance.** Selection of a minority subpopulation of bacteria that are capable of expressing resistance to the test agent may occur during the actual performance of an in vitro susceptibility test (8, 24, 53, 88). These organisms may be detected as survivors during either MBC or time-kill studies. Unlike persisters, these surviving colonies demonstrate an increase in resistance, relative to the parent strain, when retested (8, 24, 53, 88). Both selection of a preexisting resistant
subpopulation and induction of a latent resistance mechanism may result in the demonstration of phenotypic resistance. In MBC studies this will appear as an increased MBC-MIC ratio, and in time-kill studies one may observe the emergence of the resistant subpopulation as an increase in CFU per milliliter at the later (24- and 48-h) time points (53). The development of phenotypic resistance has been demonstrated in vivo and appears to be important clinically (53).

Technical factors. (i) Growth phase of inoculum. Stationary-phase cultures used to prepare the inoculum for MBC and time-kill studies will include an increased number of slowly growing or dormant cells that are not as susceptible to the test agent, especially cell wall-active agents, and will result in decreased estimates of bactericidal activity (10, 22, 30, 49, 58, 79, 81). The need for logarithmically growing rather than stationary-phase cultures when performing bactericidal studies has been stressed repeatedly in the literature to avoid underestimating the ability of an agent to kill a given strain of bacteria (30, 81).

(ii) Inoculum size. The size of the starting inoculum (CFU per milliliter) is considered the single most important variable in antimicrobial susceptibility testing (53). Both inoculum size and growth rate may independently affect determinations of bactericidal activity. It is known that tolerance to the killing effects of antimicrobial agents increases when a large inoculum is used and decreases with small inocula (28, 58, 61, 75, 79, 82). Even when log-phase cultures are used, a large inoculum approaches the stationary growth phase, with the result that the organisms may be killed more slowly. It is recommended that inocula from logarithmically growing cultures be prepared in a shaker-incubator to ensure uniformity of growth (58, 75, 79).

(iii) Insufficient contact. Organisms in broth are generally considered to be fully exposed to the antimicrobial agent in solution. This may not be entirely true depending on how the inoculum was added to the tube or well, the degree of splashing that takes place during incubation, and the composition of the tube (58). Viable organisms may adhere to the walls of the test vessel above the level of the meniscus and away from the agent in solution. Splashing at the time of inoculation and during incubation may exacerbate this condition and may wash organisms back into solution, creating a situation of variable contact with the test agent. Such adherence is more likely to occur with plastic tubes than with acid-treated borosilicate glassware or plastic microdilution trays. Delivering the inoculum below the surface of the broth test medium is recommended to minimize splashing (53, 58). Test tubes (but not microdilution trays) should be vortexed after 20 h of incubation and again immediately preceding sampling at 24 h for MBC determinations (53, 58). Likewise, when tests are done with flasks or bottles, these containers should be shaken continuously to ensure better contact between all cells and the antimicrobial agent (53).

(iv) Antimicrobial agent carryover. The determination of survivors by plating an aliquot from broth-containing tubes or wells onto an agar surface may be confounded by carryover of the antimicrobial agent, resulting in continued inhibition of growth on the agar plate and an overestimation of the killing effect (53, 58, 61, 75, 88). This is more of a problem at higher concentrations of antimicrobial agent (>16× MIC) and with larger transfer volumes. Carryover can be avoided by serially diluting or washing the sample for subculture or by spreading the sample evenly over the entire surface of the agar plate, effectively diluting out any carryover effect (53). Carryover may be assessed by streaking a sample of the test broth across an agar plate, allowing 20 min for absorption of the antimicrobial agents into the agar, and then cross-streaking with the test organism, looking for inhibition of growth at the site of the initial streak (53).

(v) Volume transferred. The volume transferred for determination of survivors must be large enough to ensure accurate determination of 99.9% killing yet not so large that drug carryover will be a problem (58). Ideally, the volume transferred should be such that at least 10 colonies are present to be counted following incubation. Assuming a final inoculum of 5×10⁵ CFU/ml, approximately 500 viable cells or CFU/ml will remain following a 99.9% kill (53, 58). Transfer of a 10- to 100-μl aliquot will result in 5 to 50 colonies on the plate following incubation. Transfer of volumes greater than 100 μl may result in drug carryover, and volumes less than 10 μl may result in too few colonies and are also subject to pipetting and sampling error (58). Accurate quantitation of the volume transferred is essential and usually requires a calibrated micropipette. Multipoint inoculators are difficult to calibrate and usually provide a transfer volume that is too small, whereas quantitative loops, even if calibrated, are not sufficiently accurate for use in bactericidal testing (1).

(vi) Choice of media. The results of both inhibitory and bactericidal testing can be influenced tremendously by the choice of the test medium. Among the variables in media known to affect the bactericidal activity of certain antimicrobial agents and microorganisms are proteins, pH, phosphates, osmolality and salt concentrations, and divalent cations (27, 58, 61, 67, 75, 76, 79). Because of these numerous and varied interactions, standardization of the test medium is desired. Cation-adjusted Mueller-Hinton broth (CAMHB) has been recommended by the NCCLS as the standard medium for broth dilution antibacterial testing (53). Supplementation of CAMHB may be necessary to grow fastidious organisms, and adjustment of calcium and magnesium ion concentrations is important when testing aminoglycosides against Pseudomonas aeruginosa. Serum supplementation of CAMHB has been suggested for the performance of bactericidal studies and clearly may influence the results (decreased killing) when highly protein-bound agents are tested (52, 53, 58, 67, 75, 76). Again, variation in medium composition and supplementation may further confound efforts to determine the clinical value of bactericidal testing (61).

In Vivo Correlates of Bactericidal Testing
The ultimate goal of any in vitro antimicrobial susceptibility test is to provide information that will lead to the administration of the optimal therapy for an infectious disease. In most instances, the clinical utility of AST has been demonstrated more clearly for predicting therapeutic failure than for predicting a successful outcome, largely because of the multiple clinical and host factors that dictate outcomes (immune reconstitution, surgical intervention, etc.) (24, 71). Given the fact that bactericidal activity is considered necessary for proper treatment of certain infections, numerous investigators have
used bactericidal testing (MBC, time-kill, and SBT) in efforts to correlate in vitro data with in vivo treatment results (7, 12, 14, 23, 38, 51, 61, 68, 74, 86, 87). Unfortunately, the criterion used to define bactericidal activity (99.9% killing of the final inoculum) is completely arbitrary and results in an all-or-nothing situation that probably has no biological or clinical significance (52, 53). Assessing the rate of bactericidal activity may have more validity in that it provides a more dynamic means of comparing different agents and different conditions than is possible with the MBC and SBT methods (7). Given the tremendous number of variables discussed above that may confound the results of any of these test methods, assessment of clinical relevance has proven difficult at best (61).

Studies of animal models of endocarditis and in vivo-infected chambers have demonstrated that therapeutic success may be predicted if the concentration of the antimicrobial agent at the site of infection exceeds the MBC for the infecting organism (53). Likewise, the serum bactericidal rate has been shown to correlate with the rapidity of sterilization of vegetations in an animal model of S. aureus endocarditis (7).

Clinical data exist supporting the importance of bactericidal regimens in the treatment of meningitis, endocarditis, osteomyelitis, and various infections in neutropenic patients (7, 12, 14, 23, 38, 51, 61, 68, 74, 86, 87). Several studies have shown that rapidly bactericidal therapy results in superior outcomes in children with either pneumococcal or gram-negative bactillary meningitis (14, 23, 38, 41, 48). Based on these reports, it appears that time-kill kinetic studies may be more useful than MIC or MBC determinations in selecting the best treatment regimen.

More limited data are available to support the use of bactericidal testing for other infections. Peak serum bactericidal titers of >1:8 suggest optimal therapy in a limited number of studies of endocarditis, bacterial infections in cancer patients, osteomyelitis, and suppurative arthritis (74, 86, 87).

Given the disparate clinical settings that have been systematically evaluated, bactericidal testing is usually not done. When it is used, the vagaries of the various methods described above suggest that use of a “standardized” method is needed to permit any sort of comparison of data over time (52, 53, 61). Testing of a specific clinical isolate and subsequent interpretation of the results should be done only in relation to clinical evidence of responsiveness or lack thereof. This occurs most often when a patient with a well-defined infection (e.g., endocarditis) fails to respond to what should be appropriate therapy, in which case a bactericidal test may help ensure that the infecting organism was killed (or not) by the agents being used (52, 53, 61). The method of choice may vary; however, increasing amounts of data suggest that time-kill studies may provide more reliable and clinically useful data than the static dilution studies (53). Importantly, bactericidal studies should not be given excessive credibility and should not be used inappropriately. Perhaps the best use of these in vitro tests is in the preclinical evaluation of a new antimicrobial agent, where in vitro and in vivo infection models can be used to establish the bactericidal nature of the agent. With this information in hand, the bactericidal activity of an agent would need to be reassessed by an in vitro test in only rare clinical situations.

**METHODS FOR DETERMINING THE MICROBICIDAL ACTIVITY OF ANTIFUNGAL AGENTS**

Given the confounding issues and variables outlined above for bactericidal testing, those interested in performing fungicidal testing would be well advised to address parallel issues in testing yeasts and moulds.

Clearly, the technical issues of inoculum size and growth phase, insufficient contact with the test agent, drug carryover, volume transferred, and medium selection cannot be avoided (9, 21, 35, 97). Likewise, biological issues such as the paradoxical effect, persisters, and phenotypic resistance are concerns for fungicidal testing as they are for bactericidal testing (see above). An additional biological issue that may affect fungicidal and bactericidal testing is the so-called postantibacterial/antifungal effect (PAE, PAFE) (2, 17, 18, 44, 84). Agents exhibiting a PAE/PAFE require extended incubation following subculture in either time-kill or MLC determinations in order to ensure the detection of slow-growing but not dead organisms (53). Additional concerns specific to mycological testing are slower growth rate, morphologic variation or complexity (unicellular yeast vs. multicellular mould), mechanism of action of the test agent, and, to some extent, stability and solubility of the test agent (6, 21, 34, 35, 37, 40, 45, 47, 66, 80). Although initial efforts at standardization of fungicidal testing have taken many of these variables into consideration, there are still major issues that have not been addressed (9, 21, 35, 37).

Given the meager data in support of the clinical utility of bactericidal testing, one must be quite skeptical about whether even if standardized, fungicidal testing will have any true clinical role. These reservations not withstanding, the use of a standardized method of fungicidal testing in preclinical studies, along with appropriate data from animal models, may be useful in positioning agents for use in selected infections and patient populations.

Although arbitrary, the use of 99.9% (or 3-log-unit decrease) kill of the final inoculum is the most stringent in vitro criterion for determining fungicidal activity. However, and as discussed below, the most convincing evidence classifying an agent as fungicidal is organ clearance of the infecting organism in a neutropenic-animal model of disseminated infection, and data generated in this fashion are arguably the “gold standard” for assessing cidality. Unfortunately, even that assessment is limited to the few organisms for which reliable models of infection have been established.

**Animal Models as a Gold Standard for Defining Fungicidal Activity**

The gold standard for demonstrating fungicidal activity is the determination of the residual fungal tissue burden in neutropenic-animal models of disseminated infection. Studies of neutropenic rabbits (Candida and Aspergillus), guinea pigs (Aspergillus), and mice (Candida), using residual fungal tissue burden in the main target sites (kidneys, liver, spleen, and lungs) as the primary end point and $C_{\text{max}}$ (peak concentration in serum), AUC (area under the concentration curve), time above the MIC or MFC, and tissue concentration as pharmacodynamic end points, have clearly documented the in vivo
fungicidal activity of the echinocandins (anidulafungin, caspofungin, and micafungin) against Candida and the lack thereof of these agents against Aspergillus despite improved survival and lung infarct score (2, 25, 32, 62, 64, 65, 72, 85). Conversely, the new triazoles (posaconazole, ravuconazole, and voriconazole), when tested in these models, show primarily fungicidal activity against Aspergillus and fungistatic (decreased organ clearance) activity against Candida (2, 11, 31, 33, 63, 72).

Similar studies with amphotericin B have documented rapid fungicidal activity against Candida, with clearing of all target organs (2, 62, 65, 85). Animal models of aspergillosis demonstrate that high rates of hyphal injury and fungal death produced by amphotericin B, voriconazole, and posaconazole are reflected by abnormal fungal morphology on histopathologic examination, a lower residual fungal burden, and parameters indicating decreased pulmonary injury (infarct scores and lung weights) as well as increased survival (2, 31–33, 63, 64, 65, 72). Conversely, although the echinocandins improve survival in neutropenic rabbits with pulmonary and disseminated aspergillosis, they have only a modest effect on the residual fungal burden of Aspergillus in tissues (25, 64, 65, 72). These agents cause dose-dependent morphologic damage of hyphal structures in tissues, which results in an impaired ability of the fungus to invade blood vessels and cause tissue infarction and death (6, 25, 64, 65, 72). However, in the absence of neutrophils, the damaged hyphal elements remain viable and are able to grow with normal morphology when the agent is withdrawn or the organs are cultured.

This type of data serves as an excellent gold standard for assessment of the fungicidal activity of candidate agents against Candida and Aspergillus. Once these general patterns are determined, it is possible to devise in vitro methods that can be used more easily and inexpensively to assess the fungicidal activity of various agents against an expanded number of Candida and Aspergillus isolates and, if indicated, for clinical purposes as well. Unfortunately, adequate models for other fungal pathogens are usually not available, thus limiting our ability to provide a similar assessment of the utility of agents against other “emerging” opportunistic fungal pathogens.

**In Vitro Methods for Determining Fungicidal Activity for Antifungal Agents against Yeasts and Moulds**

Efforts at developing standardized methods for performing time-kill and minimal fungicidal concentration (MFC [MLC]) determinations for various antifungal agents have begun and have demonstrated that by following a common procedure, various laboratories may reliably perform and interpret both types of studies with Candida and Aspergillus (9, 21, 35, 37). These approaches must be examined closely to ensure that known technical and biological variables have been addressed. Furthermore, considerable effort must be undertaken to ensure that, at the very least, agents classified as fungicidal by in vitro methods actually do clear target organs of the infecting organism in animal models of infection. Even if all of these requirements are met, there is no assurance that such in vitro testing will actually be meaningful clinically. As with bacterial testing, it is likely that such testing will play a very limited role in the management of patients with fungal infection.

**Time-kill studies.** As noted above, time-kill studies of antimicrobial agents provide a more dynamic assessment of the interaction between an antimicrobial agent and a given organism and may have greater clinical utility than the static MLC determinations. As such, time-kill studies have been used to assess the relative rate and extent of the bactericidal activity of various agents, their pharmacodynamic characteristics, and potential antagonism or synergy among agents administered concomitantly. In light of this body of data for antibacterial testing, time-kill methods have recently been applied to the study of antifungal agents, and a proposal for standardized methods for testing of Candida and nonmucoid strains of Cryptococcus neoformans has been introduced by Klepser et al. (35, 37) (Table 1; Fig. 1).

As with antibacterial studies, the proposed method for time-kill testing of yeasts takes into consideration several important variables including starting inoculum, medium, volume transferred, antifungal carryover, and agitation of the culture to ensure optimal growth and contact with the antifungal agent (Table 1). Klepser et al. adopted the arbitrary yet stringent criteria of a 99.9%, or 3-log10-unit, reduction in CFU per milliliter from the starting inoculum used to define bactericidal activity as the definition for fungicidal activity (Fig. 1). Given this definition of fungicidal activity, it was immediately apparent that the inoculum used to determine MICs as described in NCCLS document M27-A2 (0.5 × 10^3 to 2.5 × 10^5 CFU/ml) would preclude accurate determination of 99.9% killing due to both sampling error and antifungal carryover (35, 54). A higher inoculum of 1 × 10^5 to 5 × 10^5 CFU/ml was found to allow the determination of a 99.9% reduction in CFU per milliliter while using a transfer volume of 30 μl that minimized the problems of antifungal carryover. Notably, despite the higher inoculum used in time-kill studies for Candida and C. neoformans, the relative fungicidal and/or fungistatic activities determined by time-kill determinations were reflective of the overall pattern of MICs (the higher the MIC, the higher the concentration required in time-kill studies) of azoles (fluconazole and voriconazole), amphotericin B, and echinocandins (anidulafungin, caspofungin, and micafungin) (16–19, 34–37, 42, 43).

Although RPMI 1640 broth medium was used in these time-kill studies in an effort to parallel M27-A2, it is clear that the fungicidal activity of the echinocandins is significantly influenced by the choice of growth medium (16–19). Some agents are not uniformly fungicidal against Candida spp. when time-kill studies are done using RPMI medium (Fig. 1) but appear to be so when antibiotic medium 3 is used (16–19). When tested in RPMI medium, the echinocandins exhibit fungicidal activity that is concentration dependent but may vary within and among the different Candida species, whereas these agents appear to be uniformly fungicidal against Candida spp. when tested in antibiotic medium 3 (16–19).

Klepser et al. (35) did not find that agitation of the tubes or flasks during the course of time-kill studies affected the results with three different classes of antifungal agents (echinocandins, polyenes, and triazoles) and three different species of Candida (C. albicans, C. glabrata, and C. tropicalis) (Fig. 1). However, control cultures of some strains grew at an increased rate with agitation, and therefore agitation is recommended for time-kill studies of Candida (35).
With this background, time-kill studies have been used to study the fungicidal and fungistatic activities of polyenes (amphotericin B and nystatin), echinocandins, and triazoles (fluconazole and voriconazole) against Candida and C. neoformans (Fig. 1 and 2) (16–19, 34–37, 42, 43). By testing several different concentrations (multiples of the MIC) of each agent and sampling at 0, 2, 4, 8, 12, and 24 h, detailed studies of pharmacodynamic properties (determination of time- or concentration-dependent killing [PAFE]) and the rate and extent of fungicidal activity of various agents have been possible (Table 2; Fig. 2). Likewise, time-kill studies are superior to checkerboard studies for the evaluation of antifungal combinations against Candida spp. (42, 43).

This "standardized" time-kill method was examined in a seven-laboratory multicenter study to determine the intra- and interlaboratory reproducibility of the method in finding the fungicidal and fungistatic activities of amphotericin B and fluconazole against four isolates of Candida (two isolates of C. albicans and one each of C. glabrata and C. tropicalis) (37). The time-kill procedure was performed in each laboratory as described above and outlined in Table 1. Three different concentrations of each agent were tested, and experiments were conducted in triplicate over 24 h, with samples removed for colony size determination at selected time points. Both intra- and interlaboratory reproducibility data indicated good reproducibility (90 to 93%) with respect to sampling methods and time-kill interpretation for all four isolates (37). These results are encouraging and suggest that methodological standardization of time-kill studies for Candida is feasible.

Despite these positive steps, there are several unresolved issues regarding the performance of time-kill studies. Thus far, the method described by Klepser et al. (35) has not addressed two important technical issues: the growth phase of the inoculum (log versus stationary) and the specific manner by which the inoculum is added to the broth. As with bactericidal testing, one should strive to standardize these issues. Cell wall-
active agents such as the echinocandins are likely to be more fungicidal against actively growing cells than against those in stationary phase. Likewise, *C. albicans* isolates entering the stationary phase of growth appear to develop phenotypic resistance to amphotericin B (45). Subsurface delivery of the inoculum is important in bactericidal testing since splashing of the inoculum above the surface of the medium results in insufficient contact between the organism and the test agent and an underrepresentation of the cidal activity (58). A similar approach to delivery of the inoculum should be part of fungicidal studies as well.

The stability of the various antifungal agents over the course of time-kill studies is also a concern. Specifically, regrowth of *Candida* spp. at the 24-h time point has been noted with am-

![FIG. 2. Example of a time-kill plot showing the dose-dependent fungicidal activity of amphotericin B against an isolate of *C. albicans*. Control (●), 0.125× MIC (○), 0.25× MIC (▲), 0.5×MIC (●), 1×MIC (▲), 2× MIC (●), 4× MIC (+), 8× MIC (○), 16× MIC (■), and 32× MIC (□) curves are shown. Reprinted from reference 34 with permission.](http://cmr.asm.org/)
photericin B (35, 37). Rather than indicating the emergence of phenotypic resistance, retesting of these “persisters” showed the same MIC as for the parent strain and analysis of the test medium demonstrated a decrease in the concentration of amphotericin B of more than 50% at 24 h (37). Importantly, this effect was seen when the test concentration approximated the fungicidal activity of amphotericin B, itraconazole, and voriconazole against Candida spp. and Aspergillus isolates. Although they were all of which were selected for favorable growth characteristics (35, 37). Caution should be exercised in extrapolating this time-kill method to mucoid strains of Candida neoformans and to filamentous fungi until the methods are carefully evaluated with these organisms.

Regarding time-kill studies of filamentous fungi, the use of viability counts to monitor growth kinetics is not feasible with moulds due to their nonhomogeneous growth. Indeed, there are very few studies that have used time-kill methods to study moulds. Manavathu et al. (47) used a time-kill method to study the fungicidal activity of amphotericin B, itraconazole, and voriconazole against Aspergillus isolates. Although they were able to demonstrate concentration- and time-dependent decreases in the number of CFU per milliliter for each agent, they did not achieve a ≥99.9% reduction for any of the organism-drug combinations. Notably, they did not detail any problems with sampling of the control or drug-containing tubes for CFU determinations over time. Thus, time-kill studies of moulds may be possible, but further study is recommended.

Minimal fungicidal concentration determination. In contrast to time-kill studies, several studies of both new and established agents report MFCs of these agents against Candida, Aspergillus, and other fungal pathogens (9, 20, 21, 25, 29, 50, 57, 60, 62–65, 80, 83). Unfortunately, due to the lack of standardization of a method for performing and interpreting MFC determinations, a variety of approaches have been used, with predictably confusing results. For the most part, the more recent studies have all begun using the broth dilution method for MIC determinations described in NCCLS documents M27-A2 and M38-A for Candida and Aspergillus, respectively. The greatest source of variation comes in the volume sampled from the clear wells or tubes and the criterion regarding the percentage of “killed” cells that would indicate the MFC. Various reports have defined the MFC as the percent reduction in CFU per milliliter from the starting inoculum equal to or greater than 90, 95, 97, and 99.9% (9, 20, 21, 25, 29, 50, 57, 60, 62–65, 80, 83). Clearly, not all of these criteria are adequate to define fungicidal activity.

The main problem with these studies for yeasts is that by using the NCCLS M27-A2 procedure, they are constrained by an inoculum size of 0.5 × 10^4 to 2.5 × 10^5 CFU/ml. By using this inoculum size, the total number of yeast cells in the inoculated wells of a microdilution tray ranges from 100 to 500. Thus, when the MFC procedure is performed by sampling 10 µl of the clear wells onto agar plates, one is unable to determine anything more than a 90% decrease in CFU per milliliter from the starting inoculum. Even if the entire volume of the well were sampled, an accurate detection of a 99.9% decrease in CFU per milliliter would not be possible. The use of a less stringent criterion for defining the MFC poses the risk of “very major” errors by classifying an agent as fungicidal when in fact it is not.

Recently, Canton et al. (9) proposed a modification of the M27-A2 MIC procedure to allow MFC determinations for amphotericin B tested against Candida spp. To generate a method that employed the more stringent criteria of a 3-log-unit reduction, or ≥99.9% killing, these investigators used a larger inoculum of 10^4 CFU/ml and a larger sampling volume (200 µl, or the entire contents of each clear well). Antifungal carryover was avoided by subculturing onto two 90- by 15-mm
Sabouraud dextrose agar plates (100 μl/plate). Each 100-μl aliquot was spotted onto the plate and allowed to soak into the agar. After the plate was dry, it was streaked uniformly to separate cells and remove them from the drug source. Importantly, the investigators found that the use of the higher inoculum did not significantly increase the MICs over those obtained with the lower reference inoculum. Furthermore, they found that the MFCs ranged from 1× MIC to 64× MIC, with 7 of 201 isolates tested appearing “tolerant” (MFC, ≥32× MIC) to the fungicidal activity of amphotericin B. MFC for C. glabrata were all ≥2μg/ml, a concentration that exceeds safely achievable concentrations of amphotericin B in serum. Although further study is necessary, including evaluation of the echinocandins, this minor modification of the M27-A2 method may allow the determination of MFCs by using the more stringent criteria for fungicidal activity (99.9% or ≥3-log-unit reduction).

A similar lack of standardization has plagued determinations of MFCs for filamentous fungi. Again, most recent studies have used the NCCLS M38-A broth microdilution method and have been largely limited to Aspergillus spp. (21, 25, 29, 50, 60, 63–65, 80). The fact that the inoculum recommended for determining the MICs of agents tested against Aspergillus spp., 1×10⁴ to 5×10⁶ CFU/ml, is higher than that for yeasts makes determination of a 3-log-unit decrease in CFU per milliliter from the final inoculum more feasible. Nevertheless, transfer volumes ranging from 10 to 100 μl and criteria for MFC ranging from 95 to 99.9% reduction in CFU per milliliter have been employed. Most of the studies, however, were able to determine a >99% reduction in CFU per milliliter, and consistent data regarding the MFCs of amphotericin B, itraconazole, posaconazole, ravuconazole, and voriconazole against Aspergillus spp. have been reported.

Recently, Espinel-Ingroff et al. (21) described testing conditions for determination of MFC of itraconazole, posaconazole, ravuconazole, and amphotericin B against Aspergillus spp. They used NCCLS M38-A broth microdilution methods for MIC determination and, following a 48-h incubation, transferred 20 μl from each clear well onto an agar (Sabouraud dextrose agar) plate. MFCs were defined as the lowest drug dilutions that yielded fewer than three colonies (approximately 99 to 99.5% killing). All studies were performed with a panel of 15 Aspergillus isolates in three different laboratories. MFC reproducibility was good to excellent (91 to 98%) for the five agents tested in the chemically defined RPMI 1640 medium. As with yeasts, it appears that methods of determination of MFCs for Aspergillus spp. may be standardized. The use of a slightly larger transfer volume (e.g., 100 μl) may allow the determination of a 3-log-unit reduction in CFU per milliliter. The role of such a standardized method of generating MFCs as a predictor of the clinical outcome of infections due to Aspergillus spp. remains to be seen (21).

Regarding MFCs, irrespective of the end-point criteria used (e.g., 99 or 99.9% reduction in CFU per milliliter), what do we deem fungicidal? The convention for bacterial testing is an MBC of no more than 2× to 4× the MIC. “Tolerance” to the bactericidal or fungicidal activity of an agent is usually defined as an MBC of ≥32× MIC but some authors have used ≥8× MIC. This is yet another issue to be addressed. It would seem reasonable to at least compare the MFC to the achievable concentration of the various agents in serum and tissues. Given that the echinocandins and some of the new triazoles (posaconazole and ravuconazole) are highly protein bound (~90%), an adjustment for protein binding may also be considered; however, enhanced clinical relevance has not been shown for serum additives in tests of most other antimicrobial agents (27, 61).

Other measures of vitality and mortality. The methods described thus far for determination of fungicidal activity are all dependent on the enumeration of replication-competent cells following exposure to an antifungal agent. The relatively recent availability of fluorescent probes specific for either vitality or mortality, coupled with measurements of intracellular ATP and particle counts, provide a much more detailed assessment of the effect of antifungal agents on both yeasts and moulds (6, 40, 45, 63, 64). Through the use of these techniques, Liao et al. (45) have shown that cells of C. albicans exposed to amphotericin B may appear incapable of replication or metabolic activity yet still maintain a physiological state that may indicate cells capable of resuscitation once the antifungal agent is removed. Likewise, Bowman et al. (6) used similar vitality- and mortality-specific dyes to demonstrate that although caspofungin does not appear fungicidal against Aspergillus spp. by conventional replication-based determinations, it does kill the cells at the apices and branch points of hyphae. Thus, these methods are powerful tools in furthering our understanding of the action of various agents on unicellular yeasts and multicellular filamentous fungi. However, the feasibility of use in clinical testing and their relation to in vivo outcome remains to be seen. In fact, although the use of mortality-specific probes shows that caspofungin kills individual growing cells of A. fumigatus, the use of vitality-specific probes indicates that physiologically intact cells remain and are detected as a substantial residual fungal burden in target organs of experimental animals (40, 63, 64).

Relationship of In Vitro Fungicidal Measurements to In Vivo Activity of Antifungal Agents

Given the problems in establishing in vitro-in vivo correlates for bactericidal determinations, it would not be surprising to learn that such data may also be limited with respect to fungi and fungicidal determinations. Notably, the well-developed neutropenic rabbit and guinea pig models of disseminated aspergillosis and candidiasis have served to define the fungicidal activity, or lack thereof, of several antifungal agents (see above) and subsequently have also provided important in vivo correlates for fungicidal determinations (2, 11, 25, 31–33, 62–65, 72, 85). Unfortunately, clinical data are either limited or unavailable.

Animal models. As described above, the neutropenic rabbit and guinea pig models of disseminated candidiasis (rabbit) and aspergillosis (rabbit and guinea pig) have demonstrated quite clearly that the echinocandins (anidulafungin, caspofungin, and micafungin) exhibit concentration-dependent fungicidal activity against Candida but not Aspergillus, based on the residual fungal burden in target organs (2, 25, 32, 62, 64, 65, 72, 85). In contrast, the triazoles (posaconazole, ravuconazole, and voriconazole) tested in the same models are fungicidal against Aspergillus but not Candida (2, 11, 31–33, 60, 63, 72). In each
case, in vitro studies using either MFC or time-kill methods proved predictive of the in vivo response. The dose-dependent in vivo antifungal effects corresponded to the in vitro concentration-dependent fungicidal effect or lack thereof. Similarly, the additive and/or synergistic fungicidal interaction of caspofungin and voriconazole determined in vitro against *Aspergillus* was validated by demonstration of fungal clearance from organs in a guinea pig model of invasive aspergillosis (32). Thus, it appears that reasonable in vitro-in vivo correlates do exist for these newer agents (and also for amphotericin B) for either MFC or time-kill fungicidal methods applied to *Candida* and *Aspergillus*.

**Clinical data.** In contrast to the animal model correlates for fungicidal determinations, similar data do not yet exist to allow an evaluation of the clinical role of either MFC or time-kill determinations. In part, this paucity of data is due to a combination of host and clinical factors. First, the classical settings in which the need for cidal therapy of bacterial infections (e.g., endocarditis) has been documented are relatively less common with the fungal infections. Second, the overall outcome of fungal infections in the neutropenic patient is more dependent on the host immune status than on any single microbiological factor (57a). Having said this, it has been demonstrated that high amphotericin B MFCs for *Candida*, *C. neoformans*, *Aspergillus* spp., and *Trichosporon beigelii* have correlated with microbiologic failure in patients (57, 66, 70, 71, 83). Nguyen et al. (57) determined the MFC of amphotericin B for 105 isolates of *Candida* spp. from as many patients with candidemia treated with amphotericin B. The strongest predictor of microbiologic failure was a 48-h MFC of >1 μg/ml. The specificity of a break point MFC at 48 h of >1 μg/ml for microbiologic failure was 94% (68 of 72), and the positive predictive value was 78% (14 of 18) (57). These data appear promising for amphotericin B MFC determinations, especially for candidemia. Unfortunately, similar data are not yet available for the newer triazoles and echinocandins antifungal agents; however, based on the animal model correlates, it would appear that clinical correlates may exist for these agents and specific fungal infections. Standardization of MFC and time-kill methods is necessary before such studies can be attempted. Likewise, measures of clinical utility must be improved and strengthened by employing methods that measure microbial growth or fungal burden in a more sensitive, quantitative fashion (e.g., PCR) rather than simply assessing the overall survival of treated patients (25, 63–65).

**CONCLUSIONS**

Similar technical and biological variables affect both bactericidal and fungicidal testing. As with bactericidal testing, fungicidal determinations (e.g., MFC and time-kill) are likely to play a very limited clinical role, with possible application in settings of endocarditis, meningitis, septic arthritis, and osteomyelitis or in situations of poor response to normally effective agents, especially in neutropenic patients.

Standardization of methods for assessing fungicidal activity is necessary, given the great potential for variation in results. Once standardized, methods for determining the MFC or time-kill kinetics of antifungal agents are likely to have greater application, in combination with animal models of infection, in the preclinical evaluation of new agents than in the clinical setting.

Animal models of disseminated candidiasis and aspergillosis appear to offer the best in vivo setting to assess fungicidal activity and have clearly demonstrated the fungicidal activity, or lack thereof, of the new triazoles (fungicidal against *Aspergillus*) and echinocandins (fungicidal against *Candida*). Importantly, the ability of a given antifungal agent to minimize or eliminate the residual fungal burden in target organs of neutropenic rabbits or guinea pigs in a dose-dependent fashion has been shown to correlate with concentration-dependent fungicidal activity measured by either MFC or time-kill methods.

Efforts to standardize time-kill and MFC methods have just begun but, along with the in vivo animal data, show great promise for the development of uniform methods that are reproducible among laboratories and appear to be consistent with in vivo measures of fungicidal activity. Once they are standardized, the challenge, of course, will be to determine the clinical role of such testing. It is one thing to compare various agents with others in the preclinical setting. It is quite another to demonstrate that the agent with the lowest MFC or the highest rate of killing will actually prove to be superior in treating a given patient. The ability to apply these tests with confidence in the clinical setting will require considerable methodological refinement and efforts to determine their clinical relevance. It is likely, when all is said and done, that, just as with bactericidal testing, the clinical role of fungicidal testing will be very limited. Regardless, judicious application of fungicidal testing in unique clinical settings has some appeal. Such application is clearly some way off and awaits finalization of the standardization process and more extensive in vivo correlation before one can even begin to define its role clinically.

**REFERENCES**


