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Antifungal Agents

Methods and Protocols

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Preface

In the current medical era, fungal infections have emerged as an important clinical threat, with significant associated morbidity and mortality. Along with the emergence of fungal infections has come the development of antifungal resistance to existing antifungal agents and the development of agents directed at novel drug targets. Methods for evaluating such resistance patterns, mechanisms of resistance, and novel antifungal agents have all been successfully developed recently.

This volume of the Methods in Molecular Medicine series, Antifungal Agents: Methods and Protocols, is divided into three major sections covering molecular methods applied to antifungal resistance, the discovery and evaluation of new and existing antifungal agents, and host response and immunotherapy. We have avoided including methods that have been standardized, such as minimum inhibitory concentration testing, since these methods are readily available elsewhere. Many of the protocols were developed in authors’ laboratories and are provided in sufficient detail to facilitate their application and reproduction in other laboratories. For some chapters, more extensive introductory material is provided prior to the methods. In cases in which the methods are not easily applied to other organisms, alternate methods are presented separately. For example, protocols may often be applicable for yeast only where dimorphic fungi must be studied using markedly different techniques, as in animal models of candidiasis compared to aspergillosis. In other instances, the methods may apply to both yeast and dimorphic fungi with only minor methodological adaptations, such as in vitro testing of antifungal combinations of *Candida* spp. versus *Aspergillus* spp.

These methods will be of high value to clinicians, microbiologists, and molecular biologists performing research on yeasts, molds, and antifungal agents.

*Erika J. Ernst*
*P. David Rogers*
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Susceptibility Testing Methods of Antifungal Agents

Erika J. Ernst

Summary

Several methods for testing antifungal susceptibility are currently utilized. Minimum inhibitory concentrations can be tested using standardized noncommercial or commercial tests. Fungicidal testing includes either in vitro methods, such as time-kill or minimum fungicidal testing methods, or animal models. This chapter provides background information for utilizing and evaluating results obtained from antifungal susceptibility testing methods.

Key Words: Antifungal susceptibility testing; yeast; filamentous fungi.

1. Introduction

The standardization of antifungal susceptibility testing methods is crucial for the evaluation and development of antifungal drugs. It is through these methods that existing and new therapies can be compared. Although these methods have been standardized for a long time for bacteria, they only recently have been adopted for fungal pathogens. Although many clinical microbiology laboratories are comfortable with handling antibacterial specimens and implementing long-approved testing methods, the same level of comfort does not yet exist for fungi. Many of the techniques described in this text begin with the selection of organisms, including the determination of the minimum inhibitory concentrations. It is therefore important to understand the different techniques available for antifungal susceptibility testing.

Another technique for comparing the activities of compounds is the evaluation of its “idal” effects, or the ability of the drug to kill a pathogen as opposed to simply inhibiting its growth. Although the methods for evaluating a drug’s fungicidal activity have not yet been standardized, there are both in vivo and in vitro techniques for making these comparisons. It has been recognized that a need exists for standardizing these methods.
In many cases, it is more difficult to standardize methods for fungi compared with bacteria. The methods adopted for yeast may not be well suited for filamentous fungi or the methods well tested for Candida may not be appropriate for evaluating Cryptococcus spp. Despite these hurdles, some progress is being made in the standardization and application of techniques used for comparing and evaluating novel antifungal agents. This progress will prove useful in the drug development process for antifungal agents.

2. Antifungal Susceptibility Testing

Antimicrobial susceptibility testing methods for fungi recently have been standardized by the Clinical and Laboratory Standards Institutes (CLSI) (formerly known as the National Committee for Clinical Laboratory Standards [1,2]). The CLSI first published methods for antifungal susceptibility testing of Candida spp. and Cryptococcus neoformans in 1997, whereas the methods for filamentous fungi were approved in 2002. A macrodilution method for susceptibility testing was approved first, quickly followed by a microdilution method that is simpler and equally effective. It is important to note that only nonproprietary methods are standardized by the CLSI. Thus, some methods including colorimetric or E-test methods, although shown in studies to compare with the microdilution methods will not be standardized by the CLSI. Although in some instances proprietary tests may be more costly, they are often simpler and more easily implemented in laboratories with little experience working with fungi. Recently, a disk diffusion method for testing yeast has also been approved by the CLSI (3). Several important factors have been evaluated and standardized in these published guidelines including the use of synthetic media, temperature of incubation, duration of incubation, and end point definition. Several review articles have been published, providing more details than can be discussed here (4–7).

2.1. Antifungal Susceptibility Testing Methods

2.1.1. Broth Dilution

Broth dilution methods for antifungals can be performed by macrodilution or micordilution methods (2). The macrodilution method is prepared in test tubes in 1-mL volumes. This method has been established as the basis for comparing all other methods of susceptibility testing for yeast. The macrodilution method has largely been replaced by the microdilution method that is performed in a 96-well microdilution plate in volumes of 200 µL per well. Both methods use a starting inoculum on 0.5–2.5 × 10³ colony-forming units (CFU)/mL for Candida spp. and 1 × 10⁴ CFU/mL for Candida neoformans. The plates are incubated at 35°C for 48 or 72 h for Candida and C. neoformans, respec-
Susceptibility Testing Methods

The minimum inhibitory concentration (MIC) end point differs among the antifungal agents currently approved. The end point for amphotericin B is defined as the lowest concentration resulting in no visible growth, whereas the end point for theazole antifungals is when either 80 or 50% reduction in fungal growth reduction occurs for the macrodilution and microdilution methods, respectively. Trailing has been described when microdilution methods are performed on Candida species to evaluate the MIC of the azole antifungals. Trailing is a reduced but persistent growth of organism even in the presence of very high concentrations of azoles. This persistent growth can cause confusion when reading the MIC end point for the azoles against Candida species. Not all Candida display these phenomena, and it may not be apparent at 24 h but may be visible when the end point is read at 48 h. The trailing effect can be minimized by decreasing the pH of the culture medium or the addition of dextrose and starting with a higher inoculum.

The methods approved for testing common filamentous fungi include Aspergillus species, Fusarium species, Rhizopus species, Pseudallescheria byodii, and Sporothrix schenckii (1). The method has not been used for dimorphic fungi, such as Blastomyces dermatitidis, Coccidioides immitis, Histoplasma capsulatum, Penicillium marneffei, or S. schenckii. The initial challenge to the adoption of a method for filamentous fungi was the standardization of the inoculum suspension, in addition to the medium, incubation and end-point criteria. Inoculum size in addition to the antifungal had a significant impact on the testing conditions for filamentous fungi. A starting inoculum of $1 \times 10^4$ CFU/mL in RPMI 1640 broth medium was considered optimal conditions. The incubation time of 48–72 h at 35°C and an end point of at least 50% growth inhibition relative to control were selected in the approved method.

The Food and Drug Administration recently has approved a commercial broth microdilution panel for clinical use. The availability of this panel will make microdilution antifungal susceptibility testing more easily implemented in both clinical and research laboratories.

2.1.2. Colorimetric Testing

Colorimetric tests have been developed for both yeast and filamentous fungi. The YeastOne colorimetric fungal panel is a microtiter tray containing dried antifungal drugs in addition to Alamar Blue, an oxidation-reduction indicator that changes color in the presence of microbial growth. The MIC is recorded as the first well to show a change in color from pink, indicating growth, to purple or blue, indicating inhibition. This method has been correlated with the NCCLS microdilution method in several studies (8–10).
The colorimetric test used for filamentous fungi uses MTT, that is, [3-(4,5-dimethoxy-2-thiazyl)-2,5-diphenyl-2H-tetrazolium bromide] as a dye indicator to signify growth and inhibition (11). Preliminary tests indicate this as an alternative to broth microdilution.

A rapid susceptibility testing method based on a colorimetric test may allow for results in 8 h compared with 48 h needed for microdilution (12). Initial studies with this test indicate it has a high level of agreement with the NCCLS method for amphotericin B, but lower agreement for some triazole antifungals (12,13).

2.1.3. Spectrophotometric Testing

Spectrophotometric methods offer an alternative to visual interpretation of the MIC. This may reduce the subjectivity associated with the end points necessitating less than complete visual inhibition, such as with the azole and echinocandin antifungals. These methods may be particularly helpful in the case of trailing seen with the azole antifungals. Agitation of the microtiter tray before visual or spectrophotometric reading may also improve end point readings (14,15).

2.1.4. Disk Diffusion

Disk diffusion susceptibility testing offers a simple, rapid, and cost-effective method for susceptibility testing. Recently, the NCCLS approved disk diffusion susceptibility testing methods for Candida species against fluconazole and voriconazole (3). Disk diffusion methods for filamentous fungi have not been approved. The disk diffusion method provides results sooner than the microdilution method; however, only qualitative results can be obtained using this method. It is anticipated that methods for other antifungal agents and the filamentous fungi will be developed. The E-test is a commercial agar diffusion-based method for susceptibility testing. The E-test has been evaluated for testing of both yeast and filamentous fungi (16–18). It may offer a simple alternative to the microdilution method. The E-test uses a plastic strip containing an antifungal gradient. When the strip is placed on solid agar, the antifungal diffuses into the agar and inhibits microbial growth in an elliptical pattern. The intersection of the ellipse with the strip is read as the MIC. Advantages of this method over the microdilution method include the set-up time and ease in reading the end point. However, this method can be more costly compared with the microdilution method. An advantage of the E-test over the standard disk diffusion method is that the E-test provides quantitative results whereas the standard disk diffusion method only provides qualitative results. As mentioned previously, although studies indicate this method to be a good alternative to the
2.1.5. Flow Cytometry

Rapid testing of antifungal susceptibility would provide an improvement over currently available methods. Flow cytometry is an investigational method that is able to provide an MIC in as few as 2 h for amphotericin B and 6 h for fluconazole. It has been evaluated for *Candida* spp., *C. neoformans*, and *Aspergillus* spp. The test is based on the decrease in fluorescence intensity of cells stained with a membrane potential-sensitive cationic dye, after drug treatment (19–21). This method produces MIC results comparable to the microdilution and E-test methods. The major disadvantage of this method is the need for expensive laboratory equipment. However, obtaining MIC results in hours instead of days may outweigh this cost.

3. Fungicidal Testing

The ability of a compound to kill a pathogen as opposed to simply inhibiting its growth is an apparently desirable quality, particularly in the setting of decreased immunity. Although several studies have characterized the fungicidal activity of antifungal agents, there is no standardized method for doing so. The NCCLS has published guidelines for evaluating the “cidal” activity of antibacterial agents (22). Procedures are described for performing minimum bactericidal concentration, time-kill studies, and the serum bactericidal test. These tests are classified as “special” antimicrobial susceptibility tests because they are not routinely performed. However, the availability of standardized procedures allows for the comparison of results and the assessment of the clinical relevance of these results. The reader is referred to a thorough review for more details on this topic (23).

3.1. Animal Models

The neutropenic animal model of disseminated infection has long stood as the gold standard for demonstrating fungicidal activity. Residual fungal burden in target tissues such as kidney, liver, spleen, and lungs have served as the primary end point. Studies have been conducted using a variety of animals, including rabbits, guinea pigs, and mice (24–27). Combining the end point of fungal burden with pharmacokinetic end points, such as $C_{\text{max}}$, (peak concentration), AUC (area under the curve), and time above the MIC or minimum fungicidal concentration (MFC), have documented the in vivo fungicidal activity certain antifungals against specific pathogens. Detailed methods for conducting these types of studies are described in Chapter 9. It is an important element.
of the drug-development process to be able to combine susceptibility results with a clinical end point, such as the sterilization of a target tissue. These types of studies allow for the characterization of the pharmacodynamic properties of the antifungals under evaluation and may aid in the selection of dosage regimens to improve activity and decrease toxicity. Although *Candida* and *Aspergillus* animal models exist and have been used to evaluate the pharmacodynamic properties, including fungicidal activity of available and investigational drugs, appropriate models for other fungal pathogens have yet to be developed. Once useful animal models have been established, alternative and comparable in vitro models may be implemented to decrease the cost of evaluating the pharmacodynamic properties of candidate antifungal agents.

### 3.2. In Vitro Methods

Some studies have aimed at developing standardized methods for performing time-kill and MFC studies. These studies indicate that common procedures can be implemented in various laboratories with reproducible results. Similar to the time-kill studies are the in vitro models of infection. The time-kill and in vitro models of infection are particularly well-suited for evaluating the cidal activity of antifungal combinations. All of the in vitro methods have the advantage of being less expensive compared with animal model systems. Their clinical relevance also needs some validation. For example, simply determining that an agent can achieve fungicidal activity in vitro is not enough if animal models do not support that target organs are cleared of fungi or fungicidal activity occurs at concentrations that are not achievable in humans.

#### 3.2.1. Time-Kill Studies

Time-kill studies provide a more dynamic assessment of the interaction between the antimicrobial agent and a given pathogen, compared with traditional susceptibility testing. These studies are able to characterize both the rate and extent of killing because they include multiple samples taken over a given time period, usually 24–48 h. They may also be implemented to evaluate drug combinations, test for synergy and antagonism, and describe pharmacodynamic characteristics.

A proposed method for time-kill testing of *Candida* and nonmucoid strains of *Cryptococcus* has been published (28,29). This method, similar to those for bacteria and standard susceptibility tests, takes into consideration factors such as starting inoculum, medium, volume transferred, antifungal carryover, and agitation of the culture to ensure optimal growth.

To use this method to evaluate the fungicidal effects of antifungals, it is necessary to adopt criteria for cidal activity. Using criteria previously applied
Susceptibility Testing Methods

to bacteria as a basis for fungi, the strict criteria of a $3 \log_{10}$ unit or 99.9% reduction in fungal growth from the starting inoculum has been adopted. Using this definition it is recognized that the starting inoculum would need to be at least $1 - 5 \times 10^5$ CFU/mL. Using this starting inoculum and a transfer volume of 30 µL to minimize problems of antifungal carryover and assessment of fungicidal activity can be made.

3.2.2. Minimum Fungicidal Concentration

Although standardized methods have been proposed for time-kill studies, the same situation does not exist for MFC determinations. The literature contains several studies with conflicting results. Again by adopting methods originally developed for bacteria, many studies take samples from microtiter trays set up for MIC determinations and plate a volume to observe for growth. The main problem with this procedure is that the starting inoculum recommended by the NCCLS for MIC determinations is $0.5 - 2.5 \times 10^3$ CFU/mL. Using a sampling volume of 10 µL, as is the case for bacteria, but with a lower starting inoculum, one is only able to determine a 90% decrease in CFU/mL. Using the entire volume of each well would not provide an accurate detection of 99.9% reduction from the starting inoculum. Using less-stringent criteria for cidal activity increases the risk of classifying an agent as fungicidal when in fact it is not. A modification of the M27-A2 procedure, as recently proposed by Canton et al. would allow determination of the MFC based on the MIC procedure. This modification includes increasing the staring inoculum and obtaining two sampling volumes, plated separately to minimize antifungal carry over (30).

MFC determinations for filamentous fungi are also hindered by lack of standardization, although progress is being made in this area as well. Most of the studies in this area are with *Aspergillus* spp. and are based on microdilution methods. In this case the recommended starting inoculum for susceptibility testing of *Aspergillus* spp. is $1 - 5 \times 10^4$ CFU/mL. Although it is higher than for *Candida*, reaching the 3 log unit decrease in CFU/mL is still troublesome. Most studies are able to determine at least a >99% reduction in colony forming units. A recent report demonstrates very good reproducibility among three laboratories employing the same method, based upon NCCLS M38-A microdilution method, applied to a panel of 15 *Aspergillus* isolates. This report provides very promising evidence that standardized methods for MFC testing can be developed for filamentous fungi (31).

3.2.3. Tests of Vitality

All of the techniques for evaluating fungicidal activity have included quantitation of CFUs. Recently, fluorescent probes have been developed which...
are specific for either mortality or vitality. These probes along with other measurements of replication and particle counts may be able to provide a more detailed description of the effects of antifungal agents on yeasts and molds. Through the use of specific dyes, it had been demonstrated that caspofungin, despite an apparent lack of fungicidal activity against *Aspergillus* using conventional methods, is cidal to apical and branching hyphae. Further studies have shown that although caspofungin is able to kill individual *Aspergillus* cells, some cells remain and are detected as residual fungal burden in experimental animal models (24,32,33).

4. Conclusions

Antifungal susceptibility testing has advanced steadily, with standardized methods from the NCCLS now available for yeast and filamentous fungi. Other commercial testing methods that are easily implemented and interpreted are also available. Some progress has been made in the automation of susceptibility testing and we may even see a more rapid method available. Despite this progress, methods for dimorphic yeasts and molds remain elusive. Some potentially reproducible methods have also been published for fungicidal testing; however, standardization lags behind that for susceptibility testing. The definitive link between in vitro results of susceptibility and vitality and clinical success and failure has yet to be made. Even as much progress has been made and we have come a long way in the development of techniques and the standardization of antifungal testing methods, there is still more work to be done.

References


Deoxyribonucleic Acid Fingerprinting Methods for Candida Species

Shawn R. Lockhart, Claude Pujol, Andrew R. Dodgson, and David R. Soll

Summary

A number of genetic fingerprinting methods have evolved to analyze the population structure and to perform epidemiological and etiological studies of infectious fungi. These methods include multilocus enzyme electrophoresis, restriction fragment-length polymorphism using complex probes, random amplification of polymorphic DNA, and multilocus sequence typing, which are described in this chapter.

Key Words: DNA fingerprinting; restriction fragment-length polymorphism (RFLP); complex probes; random amplification of polymorphic DNA (RAPD); multilocus sequence typing (MLST).

1. Introduction

Genetic fingerprinting has become an indispensable tool in understanding the population structure, epidemiology, and etiology of fungal diseases (1,2). Several effective methods have evolved for genetic fingerprinting, including multilocus enzyme electrophoresis (MLEE), restriction fragment-length polymorphism (RFLP) with or without a probe, random amplification of polymorphic deoxyribonucleic acid (RAPD), multilocus sequence typing (MLST), and a variety of other methods. All but the first also are referred to as DNA-fingerprinting methods. Several important points must be kept in mind concerning genetic fingerprinting (1). First, the method must provide the resolution necessary to answer the question(s) posed. Second, the method must be verified for such resolution by comparison with an unrelated genetic fingerprinting method. Third, the method must be economical, specifically for large epidemiological questions, and within the capabilities of the experimenter. Finally, the data should be comparable with similar data collected by others. Here, we describe
three DNA fingerprinting methods that are both popular and generally effective: RFLP with probe, RAPD, and MLST.

2. Materials

2.1. Preparation of Genomic DNA

1. Yeast peptone dextrose (YPD) medium: 1% yeast extract, 2% peptone, 2% dextrose.
2. Sorbitol potassium phosphate (SPP): 1 M sorbitol, 50 mM potassium phosphate, pH 7.4. Make 50 mM of potassium phosphate by mixing 40.1 mL of 1 M K$_2$HPO$_4$ with 9.9 mL of 1 M KH$_2$PO$_4$ in 1 L of total volume.
3. Zymolyase solution: 100 mg of Zymolyase 20T (Seikagaku America, Ijamsville, MD) in 800 µL of 50 mM sodium phosphate, pH 6.5, and 50% glycerol.
4. Bursting buffer: 50 mM Tris-HCl pH 7.4, 20 mM ethylene diamine tetraacetic acid (EDTA).
5. 10% Sodium dodecyl sulfate (SDS).
6. 5 M Potassium acetate.
7. 1:1 Phenol/chloroform.
8. Isopropanol.
9. Absolute ethanol.
10. TE: 10 mM Tris-HCl, pH 7.5; 1 mM EDTA.
11. 10 mg/mL RNase A (Sigma, St. Louis, MO).

2.2. Probing a Genomic DNA Southern Blot

1. DNA molecular weight markers (Promega, Madison, WI).
2. 1X TBE: 10X TBE is 108 g of Tris base, 55 g of boric acid, and 20 mL of 0.5 M EDTA, pH 8.0, in 1 L of total volume.
3. Random Primer Labeling Kit, (Life Technologies, Gaithersburg, MD).
5. DNA loading dye: 0.25% bromophenol blue, 0.25% xylene cyanol, 15% Ficoll type 400 in sterile water.
6. EcoRI restriction enzyme.
9. Calf thymus DNA: prepare a 10 mg/mL solution by dissolving calf thymus DNA (sodium salt; Sigma) in sterile water. Shear the DNA by sonicating at 50 W for 15-s bursts, 5–10 bursts (Sonics and Materials, Inc., Danbury, CT). Check the fragment size by running a 1-µL aliquot on an agarose gel. The average size fragment should be <2 kb in length.
10. 20X SSPE: 3.6 M NaCl, 0.2M NaH$_2$PO$_4$, and 20 mM EDTA. Adjust pH to 7.0.
11. Hybridization buffer: 5X SSPE, 5% dextran sulfate, 0.3% SDS. Store at 20°C.
12. Wash solution: 2X SSPE, 0.2% SDS.
2.3. Fingerprinting Genomic DNA by MLST

1. Taq DNA Polymerase and appropriate buffer (Invitrogen, Carlsbad, CA).
2. 2 mM (each) Deoxynucleotide 5'-triphosphate (dNTPs) (deoxyadenosine 5'-triphosphate, deoxythymidine 5'-triphosphate, deoxycytidine 5'-triphosphate [dCTP], and deoxyguanosine 5'-triphosphate).
3. 2 nM Oligonucleotide primers.
4. 50 mM Magnesium chloride.
5. Sterile distilled water.
6. 5X sequencing buffer: 80 mM Tris-HCl, pH 9.0, 2 mM magnesium chloride.
7. Commercial kit for polymerase chain reaction (PCR) product purification (e.g., Genelute PCR Clean Up Kit, Sigma).
9. Absolute ethanol.
10. 3 M Sodium acetate.
11. GlycoBlue (3.75 g/L) coprecipitant (Ambion, Austin, TX).

2.4. Fingerprinting Genomic DNA by RAPD

1. Taq DNA Polymerase and appropriate buffer (Invitrogen).
2. 0.2 mM (each) dNTPs (deoxyadenosine 5'-triphosphate, deoxythymidine 5'-triphosphate, dCTP, and deoxyguanosine 5'-triphosphate).
3. RAPD 10-mer primer kits (Operon Technologies, Alameda, CA).
4. 1X TBE: 10X TBE is 108 g of Tris-HCl base, 55 g of boric acid, and 20 mL of 0.5 M EDTA pH 8.0, in 1 L of total volume.
6. DNA loading dye: 0.25% bromophenol blue, 0.25% xylene cyanol, 15% Ficoll type 400 in sterile water.
7. 40 µg/L of ethidium bromide solution.

3. Methods

3.1. RFLP With a Probe

RFLP with a complex DNA fingerprinting probe has been demonstrated to be a very effective method for analyzing large samples, providing information relevant to population structure, as well as microevolution (1,3). This protocol is performed in two steps. The first involves separating genomic DNA on an agarose gel and then fixing it to a nylon membrane. The second involves hybridization with a DNA probe. The result is a complex pattern that is amenable to computer analysis. The DNA probe must either be available for the species of interest or a DNA probe must be isolated, characterized and confirmed as an accurate measure of genetic distance (see Note 1 [4–9]).
3.1.1. Preparation of Genomic DNA

1. Grow a 2-mL culture of *Candida* spp. in YPD broth to stationary phase (10^8 cells/mL). Harvest the cells by centrifugation at 15,000g in a Microfuge for 3 min and wash the pellet one time with 1 mL of sterile water (see Note 2).

2. Wash the pellet once with 0.7 mL of SPP. Resuspend the pellet in 0.6 mL of SPP containing 0.1% β-mercaptoethanol. Add 15 µL of Zymolyase solution and incubate the cell suspension for 30–90 min at 37°C.

3. Assess spheroplasting by microscopic examination and by lysing 10 µL of cells on a microscope slide with 1 µL of 10% potassium hydroxide. Spheroplasts lyse, but cells with intact walls do not. When >80% of the cells have become spheroplasts, pellet them at 15,000g for 5 min at room temperature (see Note 3).

4. Wash the spheroplast pellet once with 0.7 mL SPP. Resuspend the pellet in 0.6 mL of bursting buffer by repeatedly drawing the pellet into a 1 mL pipet until the solution is homogeneous. Do not vortex! Add 60 µL of 10% SDS and incubate for 30 min at 65°C. Add 240 µL of 5M potassium acetate, shake the tube gently by hand, and incubate for 1 h on ice.

5. Centrifuge the cell lysates at 15,000g for 10 min at 4°C. Recover the supernatant and extract once with an equal volume of phenol/chloroform. Do not vortex. Precipitate the nucleic acids with an equal volume of isopropanol. Wash twice with 0.7 mL of 75% ethanol. Do not centrifuge the DNA pellet for more than 30 s.

6. Dry the nucleic acid pellet on the bench top and resuspend it in 100 µL of TE.

7. Remove the contaminating RNA by adding 2 µL of RNase A and incubating for 1 h at 37°C. If desired, extract once with an equal volume of phenol/chloroform and precipitate as in step 5. Resuspend the pellet in 100 µL of TE.

3.1.2. Probing a Genomic DNA Southern Blot

1. Digest 3 µg of the *Candida* genomic DNA with EcoRI or another appropriate restriction enzyme in a 30-µL final volume following the manufacturers instructions. Use a threefold excess of enzyme and allow the reactions to take place for at least 4 h. Add 3 µL of DNA loading dye to each reaction.

2. Separate the genomic DNA on a 0.8% agarose 1X TBE gel run at 120V until the bromophenol blue dye has migrated 18 cm from the origin. Run the same strain in the outside lanes of each gel so that the gels can be later calibrated to each other (see Note 4).

3. Transfer the gel to a Hybond N+ membrane by Southern blotting. After Southern blotting, fix the DNA to the Hybond N+ membrane by exposing the membrane, DNA side up, to ultraviolet light for 3.5 min (see Note 5).

4. Place each blot into a separate-heat sealable bag with 5 mL of prewarmed hybridization buffer containing 0.1 mg/mL heat-denatured calf thymus DNA. It is desirable to seal the bag so that it is only slightly larger than the blot; this will concentrate the hybridization buffer as well as the probe. Prehybridize for 6–16 h at 65°C in a shaking water bath.
5. Prepare $^{32}$P-dCTP labeled DNA probe following the instructions provided by the manufacturer of the random primer labeling kit. Pass the probe over a Sephadex G-50 column to remove any unincorporated labeled nucleotides and count the finished probe in a scintillation counter. Add approx $5 \times 10^6$ cpm of labeled probe to the blot without changing the hybridization buffer. Let the blot hybridize overnight.

6. Pour off the hybridization buffer and probe and wash the blot 3 × 20 min in 500 mL of wash solution at 45°C in a sealed plastic container in a shaking water bath. If multiple blots were hybridized, they can be washed together.

7. Seal each blot in plastic wrap and expose to autoradiographic film for 1–3 d depending on the strength of the radioactive signal.

8. As long as the blot has not dried completely, it can be stripped of radioactive label and used again. These blots can be stored in plastic wrap at room temperature.

3.1.3 Analyzing the Repetitive DNA Probe Data

For Southern blot hybridization, patterns generated by moderately repetitive sequences, two similarity coefficients ($S_{AB}$) are useful, one based on the position and intensity of bands and one based on position alone.

1. $S_{AB}$ based on position and intensity:

$$S_{AB} = \frac{\left(\sum_{i=1}^{k} (a_i + b_i - |a_i - b_i|)\right)}{\sum_{i=1}^{k} (a_i - b_i)}$$

where $a_i$ and $b_i$ are the intensities of bands in patterns A and B, respectively, and $k$ is the number of bands. If the patterns of strains A and B are identical (all bands are matched in size [in kilobases according to their migration] and of the same intensity), the $S_{AB}$ will be 1.00. If no bands match, the $S_{AB}$ will be 0.00. Increasing matches with increasingly comparable intensities results in $S_{AB}$ ranging from 0.01 to 0.99.

2. $S_{aB}$ based on position alone:

$$S_{aB} = \frac{2E}{2E + a + b}$$

where $E$ is the number of bands in patterns A and B, which are the same size, $a$ is the number of bands in pattern A with no correlate in pattern B, and $b$ is the number of bands in pattern B with no correlates in pattern A. Again, this value will vary according to the complexity of the patterns that are compared. In the case of Candida albicans fingerprinted with the moderately repetitive probe Ca3, the average $S_{AB}$ for unrelated isolates is $0.69 \pm 0.11$ (3). This average will vary according to the complexity of the patterns that are compared.
The $S_{AB}$ can be computed between every pair of isolates and a matrix of similarity coefficients generated. Arbitrary thresholds can be assigned for unrelated, moderately related, and highly related patterns, but the best way to assess relatedness for a collection of independent isolates is through the genesis of a dendrogram based on $S_{AB}$s. Dendrograms are most easily generated based on the unweighted pair group method (10).

### 3.2. Multilocus Sequence Typing

#### 3.2.1. Choice of Loci To Be Used

MLST is a new, although effective, methodology. Where information is already available on the variability of genes or their protein products for a certain species, it may be possible to select loci to use for MLST directly. However, if there is no information on variability, it is necessary to screen a number of genes in a panel of unrelated isolates to determine which are suitable for use with MLST. It is important to choose genes that are not under selective pressure, as they will bias any results obtained for population genetic analysis. For this reason genes known to code for virulence factors or resistance determinants should not be included. The number of loci to be used in the final scheme will depend on the variability seen and the level of discrimination required, but as a guide, six to eight should be sufficient (see Note 6 [11,12]).

#### 3.2.2. PCR Primer Design

Primers can be designed using a variety of commercial packages, such as PrimerSelect (Dnastar, Inc., Madison, WI). Primers for a particular gene should be separated by between 500 and 650 bp to allow for a 450–600-bp sequence to be obtained. Although primers may be designed that fall within noncoding regions, they must amplify enough coding DNA for analysis, as only coding DNA sequence is deemed useful for analysis. Desirable features of primer pairs include, similar melting temperatures ($T_m$) for both primers in the pair and relatively high $T_m$ (as primers with low $T_m$ will require low annealing temperatures to be used, resulting in a greater likelihood of nonspecific annealing and spurious product formation). If the variability of the sequence of the locus in question is known, primers should be designed to be within conserved regions of the gene and to encompass variable regions (see Note 7).

#### 3.2.3. Amplification of Desired Loci

1. In a microfuge tube, mix 2.5 µL of 10X PCR buffer, 1 µL of dNTPs (2 mM each), 2.5 µL of each PCR primer (2 nM), 1 µL of MgCl$_2$ (50 mM), 0.2 µL of Taq DNA polymerase (5 U/µL), and 15.8 µL of sterile distilled water with 1 ng of template DNA (in 1 µL).
2. Amplify the target DNA in a thermal cycler using the following reaction conditions: 7 min at 94°C, 30 cycles of: 1 min at 94°C, 1 min at an annealing temperature appropriate to the primers used (typically 50–55°C) and 1 min at 74°C. Finish with one 10-min cycle at 74°C.
3. Purify the PCR product following the manufacturers instructions (Genelute PCR Clean Up Kit, Sigma).

3.2.4. Sequencing

All loci should be sequenced in both the forward and reverse directions. The same primers as those used for the PCRs may be used.

1. Perform the sequencing reactions in a 20-µL volume with 1.5-µL oligonucleotide primer (2 nM), 25-ng template, 4 µL of BigDye Terminator Cycle Sequencing Ready Reaction Mix, and 2 µL of 5X sequencing buffer.
2. The thermal cycling conditions are as follows: 4 min at 96°C followed by 25 cycles of 5 s at 96°C and 4 min 5 s at 60°C.
3. Remove excess dye by ethanol precipitation. Place the 20 µL of sequencing reaction product in a fresh 1.5-mL tube and add 80 µL of water, 300 µL of 100% ethanol, 5 µL of 3M sodium acetate, and 2 µL of GlycoBlue (3.75 g/L) coprecipitant. Incubate for 30 min at room temperature before pelleting by centrifugation for 20 min at 15,000g in a microfuge. Wash the pellet with 500 mL of 70% ethanol for 15 min, followed by centrifugation at 15,000g for 20 min. Remove the supernatant and dry the pellet at 100°C.

The reaction products may be analyzed using an automated DNA sequencer according to the manufacturer’s instructions (see Note 8).

3.3. Random Amplification of Polymorphic DNA

3.3.1. Principle

The RAPD approach is a frequently used method and is particularly effective for fingerprinting organisms for which DNA sequence data is limited or unavailable (13,14). In this method, genomic DNA is amplified by PCR using single, short oligonucleotide primers (10 bp). The polymorphisms analyzed consist of differences in the size of the DNA fragments amplified between samples. The choice of the primers used is usually empirical. Ideally, for a given micro-organisms, a panel of 20–40 primers is tested on a limited collection (3 or 4 isolates) and the best primers, the ones giving polymorphic bands that can be analyzed with no ambiguity and are reproducible, are selected to analyze larger collections (see Notes 9–11).
3.3.2. RAPD-PCR Amplification and Banding Pattern Visualization

All the primers used should be analyzed after identical PCR protocols. It is usually easier to test different primers rather than to try to optimize the conditions for specific primers.

1. Prepare the template DNA following the instructions provided in Subheading 3.1.1.
2. PCR is performed in 0.5-mL microcentrifuge tubes containing 25 µL of the following reaction mixture: 1 ng of genomic DNA; 2.5 µL of 10X buffer provided for the Taq DNA polymerase used; 1 U of Taq polymerase; 0.2 mM (each) dNTPs; 0.4 µM of one of the primers used, and sterile water to make up the volume (see Notes 12–14).
3. DNA amplifications are performed in a thermal cycler programmed for 45 cycles of 1 min at 94°C, 1 min at 36°C, and 2 min at 73°C, followed by a final 10-min step at 73°C.
4. Add 3 µL of DNA loading dye solution to each PCR.
5. Separate amplification products on a 1.5% agarose 1X TBE gel run at 110 V for 4 h so that the bromophenol blue marker dye migrates approx 10 cm.
6. Stain the gel with ethidium bromide by placing the gel in a solution of ethidium bromide for 1 h with agitation.
7. The bands are visualized using an ultraviolet light box and the pattern is recorded photographically (see Notes 15 and 16).
8. The equation given in Subheading 3.1.3., step 2 can be used for analysis of DNA bands generated by RAPD.

4. Notes

1. Good complex probes are available for a number of Candida species. Check the literature for availability of the probe of choice. Probes can also be manufactured for any species as long as good high-molecular weight DNA can be made from the organism (9). Protocols can be found in Girardin et al. (4), Joly et al. (5,7), Lockhart et al. (6,9), and Enger et al. (8).
2. This general methodology can be used for any species of fungi as long as good high-molecular weight DNA can be made from the organism.
3. If the yeast cells are not forming spheroplasts very efficiently, then β-glucuronidase or Novozyme (both from Sigma) can be tried instead of or in addition to Zymolyase 20T.
4. Based on the average size fragment to which the probe hybridizes, the percentage of agarose in the Southern blot gels can be adjusted to separate or compact the hybridization bands.
5. Hybond N+ membrane is preferred for Southern blotting because it is easy to strip of radioactivity and reprobe with a second probe. Because it is nylon, it is durable.
6. The methodology described is suitable for fingerprinting species with haploid genomes. Though it may be used for diploid species, some additional steps may
enhance its utility. It can be anticipated that some of the loci studied in diploid species will be heterozygous. By sequencing direct from PCR products, these heterozygosities will be shown by double peaks in the sequencing traces. Thus, where heterozygosities exist at multiple nucleotide sites in any particular locus, assignment of individual alleles is not possible. This may be overcome by cloning the PCR product into a suitable vector and sequencing again from the vector. As only one of the alleles will be present in the vector, its sequence may then be determined and the sequence of the other allele may then be inferred by deduction.

7. MLST was originally designed for use with bacteria. The high numbers of polymorphisms found within bacterial species meant that only coding DNA sequences were used in these schemes, as the use of the even more polymorphic noncoding sequences may have led to homoplasy (the presence of identical characters in distinct phylogenetic lineages that are not acquired by descent, but rather through convergence, parallelism or reversion) complicating the results obtained. However, for fungi where the differences within a species are much less marked, this may not be the case, and noncoding sequences may have a role to play.

8. A number of software packages exist for the analysis of the data obtained by MLST. Some of these may be downloaded for free at www.mLst.net. These include BURST, which uses the allele data to assess the relationships between sequence types; a program for the calculation of the index of association, and thus assessment of the likelihood of recombination within the population; and NRDB, which compares sequence data in order to assign alleles. Alternatively, the START package (available for free at http://outbreak.ceid.ox.ac.uk/software.htm) includes BURST, and index of association calculation, as well as a number of other features, such as the construction of dendrograms, tests for selective pressure, and the ability to export data in formats useable by other packages. The free package PHYLIP (http://evolution.genetics.washington.edu/phylip.html) is useful for phylogenetic analysis and the construction of dendrograms. The commercial package PAUP may be used for population genetic analysis (http://paup.csit.fsu.edu/). The commercially available Bionumerics (Applied Maths, Austin, TX) package is useful for the construction of databases containing sequence data, and the construction of dendrograms.

9. The power of this method increases with the number of primers used and the number of polymorphic bands analyzed per micro-organism. We recommend that eight primers or a total of 15–20 polymorphic bands be used to obtain data useful for cluster analysis. This may vary for any given species.

10. 10-mer custom oligonucleotides may be purchased from different companies. However, special attention should be paid to the purity of the oligonucleotides. High-performance liquid chromatography or polyacrylamide gel electrophoresis purification will be necessary for reproducible results. We found that the panels of RAPD primer kits commercialized by Operon Technologies represent a good alternative. Their panels of 1200 available primers have been selected for sequences containing a G+C content of 60–70% with no self-complementary ends. The majority of these primers work well with the PCR conditions proposed here, and for a number of different species.
11. A lack of reproducibility among laboratories, but also within the same laboratory, has somewhat hindered the use of the RAPD method. The banding patterns have been shown to be strongly affected by even slight differences in the PCR procedure (15–18). These problems can be circumvented, at least for intralaboratory reproducibility, by controlling carefully PCR procedures and the following recommendations.

12. Always use the same *Taq* DNA polymerase.

13. The stability of the patterns may depend on the concentration of the template DNA used. To test for this possibility, different concentrations of template DNA should be tested. Primers that generate patterns that are very sensitive to the template concentration should be discarded.

14. Control DNAs that have already been characterized for a given primer should always be run in parallel with new samples.

15. Independent DNA extractions of the same isolates should show the same patterns. If this is not the case, the primers showing differences should be discarded.

16. Avoid analyzing all of the bands obtained. Some of the bands obtained with a primer may be reproducible, whereas others will not be. In general, most of the bands that do not show reproducibility are low-intensity bands. One must be cognizant of this possible pitfall in analyzing such bands.

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**References**


CARE-2 Fingerprinting of Candida albicans Isolates

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Summary

The emergence of resistance to antifungal drugs in medically important fungi has become a significant problem in recent years. Probably the best-studied example is the development of resistance to the widely used antifungal agent fluconazole in the yeast Candida albicans. The availability of matched series of clinical isolates representing the same strain in which drug resistance developed over time has provided opportunities to detect cellular alterations that are correlated with drug resistance. We describe a method for DNA fingerprinting of C. albicans isolates based on Southern hybridization of genomic DNA with the C. albicans-specific repetitive DNA element CARE-2. Molecular typing with CARE-2 permits highly reliable discrimination of unrelated strains to ascertain that serial isolates recovered from individual patients indeed represent the same C. albicans strain.

Key Words: Candida albicans; DNA fingerprinting; drug resistance; fluconazole; repetitive element; strain typing.

1. Introduction

Candida albicans isolates are highly heterogeneous. Therefore, genetic and phenotypic differences between a drug-resistant isolate and a drug-sensitive isolate, for example, differences in the amino acid sequence of a drug target enzyme or in the expression of certain genes, are not necessarily linked to the resistance phenotype but may simply reflect natural variation. In contrast, if serial isolates from a single patient, in which drug resistance developed over time, all represent derivatives of the same C. albicans strain (so-called matched isolates [1]), it is more likely that alterations observed in the resistant isolates are directly responsible for the resistance phenotype or represent changes that compensate for adverse physiological effects of a particular resistance mechanism. To identify such matched isolates, it is necessary to distinguish unrelated strains by a reliable fingerprinting method. There are many different typing methods available for C. albicans, but Southern hybridization with moderately...
repetitive DNA elements has been shown to be the most powerful technique (2). One such element is the *C. albicans*-specific repetitive DNA element 2 (CARE-2), which was originally described by Lasker et al. (3) and has been shown to have the highest discriminatory power to distinguish unrelated *C. albicans* isolates from each other (4). *C. albicans* strains usually contain 10–15 CARE-2 copies, which are located on most, if not all, *C. albicans* chromosomes. When genomic DNA is digested with a restriction enzyme, the sizes of CARE-2-containing fragments differ among strains because of strain-specific restriction site polymorphisms, such that a unique banding pattern is produced in Southern hybridizations. *Eco*RI is the preferred enzyme because there is a single *Eco*RI site within the CARE-2 sequence, resulting in two hybridizing DNA fragments of variable sizes for each CARE-2 element present in the genome and thereby increasing the complexity of the fingerprint pattern. An example of a CARE-2 fingerprint analysis of different *C. albicans* isolates is shown in Fig. 1. In the following section, we describe in detail the procedure we use for CARE-2 fingerprinting of *C. albicans*.

## 2. Materials

1. Yeast peptone dextrose (YPD) medium: 2% peptone, 1% yeast extract, 2% glucose.
2. 1 M Sorbitol.
3. Lysis buffer: 1 M sorbitol; 100 mM sodium citrate, pH 5.8; 50 mM ethylene diamine tetraacetic acid (EDTA), pH 8.0; 2% β-mercaptoethanol; and 500 U/mL lyticase (Sigma Aldrich, St. Louis, MO).
4. Proteinase buffer: 10 mM Tris-HCl, pH 7.5; 50 mM EDTA, pH 7.5; 0.5% sodium dodecyl sulfate (SDS); 1 mg/mL proteinase K (Sigma Aldrich).
7. Isopropanol.
8. 70% Ethanol.
9. TE buffer: 10 mM Tris-Cl, pH 7.5; 1 mM EDTA.
10. RNase A (10 mg/mL).
11. Double-distilled H2O.
12. Restriction enzyme *Eco*RI and buffer from supplier.
13. 6X loading buffer: 0.25% bromophenol blue, 0.25% xylene cyanol, 15% Ficoll type 400.
14. 50X TAE buffer: 242 g of Tris, 57.1 mL of acetic acid, and 100 mL of 0.5 M EDTA, pH 8.0; add H2O to 1 L.
15. Ethidium bromide staining solution (5 µg/mL).
19. 20X SSC (3 M NaCl, 0.3 M sodium citrate).
Fig. 1. CARE-2 fingerprint pattern of serial Candida albicans isolates obtained from different infection episodes in a single patient. The patient was infected by two different C. albicans strains identifiable by their fingerprint pattern (A vs B). Subtle differences in the fingerprint pattern are also seen in some isolates of the strain with the B pattern (labeled B1, B2, and B3, and marked by the arrows). These genomic alterations reflect micro-evolution of the strain over time.

20. Washing buffer I: 0.5X SSC, 0.4% SDS, 6 M urea.
21. Washing buffer II: 2X SSC.
22. Solution A: 0.25 M HCL.
23. Solution B: 1.5 M NaCl, 0.5 M NaOH.
24. Solution C: 1.5 M NaCl, 0.5 M Tris-Cl pH 5.1.
25. 0.4 N NaOH.
26. 0.2 M Tris-HCl pH 7.5.
27. Ultraviolet (UV) illuminator (UV Stratalinker 1800, Stratagene, Gaithersburg, MD).
29. Enhanced chemiluminescence (ECL) kit (ECL-labeling and detection-kit, Amersham, Braunschweig, Germany).
30. GeneClean kit (GeneClean III Kit, Q-BIOgene, Heidelberg Germany).
3. Methods

3.1. Isolation of High-Quality Genomic DNA From C. albicans

1. Inoculate a C. albicans colony from an agar plate into an Erlenmeyer flask with 10 mL of YPD medium and grow the cells for 15 h (overnight) at 30°C on a rotary shaker to obtain a dense culture.
2. Pellet the cells by centrifugation for 5 min in a plastic tube at 4600g. Remove the supernatant with a 10-mL glass pipet.
3. Resuspend the cells in 1 mL of 1 M sorbitol, transfer to a 2 mL Eppendorf cap, and pellet the cells by centrifugation for 2 min at 16,000g.
4. Resuspend the washed cells in 1 mL of lysis buffer and incubate for 45 min at 37°C.
5. Pellet the protoplasts by centrifugation for 5 min at 4600g, resuspend them in 800 µL of proteinase buffer, and incubate for 30 min at 60°C.
6. Add 800 µL of phenol:chloroform:isoamyl alcohol, mix thoroughly by vigorously shaking the tubes for 2 min, and centrifuge for 1 min at 16,000g to separate the phases.
7. Transfer 800 µL of the upper, aqueous phase to a new 2-mL Eppendorf cap containing 800 µL of phenol:chloroform:isoamyl alcohol, mix thoroughly by shaking the tubes for 2 min, and centrifuge for 1 min at 16,000g to separate the phases.
8. Transfer 700 µL of the upper, aqueous phase to a new 2-mL Eppendorf cap containing 700 µL of phenol:chloroform:isoamyl alcohol, mix thoroughly by shaking the tubes for 2 min, and centrifuge for 1 min at 16,000g to separate the phases.
9. Transfer 600 µL of the upper, aqueous phase to a 1.5-mL Eppendorf cap containing 600 µL of isopropanol, mix briefly to precipitate the DNA, and pellet the DNA by centrifugation for 5 min at 16,000g.
10. Carefully remove the supernatant with a micropipet. Wash the DNA by adding 600 µL of 70% ethanol and centrifuge for 2 min at 16,000g.
11. Carefully remove the supernatant. Air dry the DNA for 10 min.
12. Completely dissolve the DNA in 200 µL of TE buffer by repeatedly pipetting it up and down.
13. Add 2 µL of RNase A solution, mix, and incubate for 30 min at 37°C.
14. Add 200 µL of phenol:chloroform:isoamyl alcohol, mix thoroughly by shaking the tubes for 2 min, and centrifuge for 1 min at 16,000g to separate the phases.
15. Transfer the upper, aqueous phase to a new 1.5-mL Eppendorf cap containing 200 µL of chloroform:isoamyl alcohol, mix thoroughly by shaking the tubes for 2 min, and centrifuge for 1 min at 16,000g to separate the phases.
16. Transfer the upper, aqueous phase to a new 1.5-mL Eppendorf cap containing 200 µL isopropanol (−20°C), mix briefly, and pellet the DNA by centrifugation for 5 min at 16,000g.
17. Carefully remove the supernatant. Add 200 µL of 70% ethanol and centrifuge for 2 min at 16,000g.
18. Carefully remove the supernatant and air dry the DNA for 10 min.
19. Thoroughly dissolve the DNA in 100 µL of double-distilled H₂O by repeatedly pipetting up and down and keep it on ice.
20. To determine the DNA concentration, dilute 10 µL of the DNA solution in 390 µL of double-distilled H₂O (40-fold) and measure the absorbance at 260 nm and 280 nm. An AD₂₆₀ of 1 corresponds to 50 µg/mL DNA (the ratio AD₂₆₀/AD₂₈₀ must be at least 1.8 for sufficiently pure DNA). The DNA yield using this protocol should be at least 200 µg. The DNA can be stored at −20°C.

3.2. DNA Digestion and Southern Hybridization

Any standard protocol can be used for the Southern hybridization analysis. In our laboratory we use the ECL labeling and detection kit from Amersham. The procedure is described in the following section:

1. For each C. albicans strain, set up a DNA digestion in a 30-µL reaction volume using 15 µg of genomic DNA, 15 units of EcoRI and the appropriate restriction enzyme buffer from the supplier. Incubate the reactions overnight at 37°C.
2. Stop the reactions by mixing with 6 µL of 6X loading buffer, load the samples onto a 1% agarose gel, and run the gel at 40 V for 16 h in 1X TAE buffer. Include a DNA size marker in the first lane of the gel.
3. Stain the gel for 15 min in a tray with ethidium bromide solution (ethidium bromide is toxic, always wear gloves when handling it!). Make a photograph of the stained gel under UV light (wear UV-protection glasses!), with a ruler placed alongside the marker lane to determine the position of size markers and to document equal loading.
4. Prepare a nylon membrane the size of the gel, rinse it for 1–2 min in a tray with double-distilled H₂O and then for 1–2 min with 20X SSC, and place it on the vacuum blotter and put the mask above it.
5. Place the gel on the nylon membrane (the gel must be slightly larger than the free area of the mask on all sides) and cover it with solution A. Apply vacuum (set to 50) and depurinate the DNA for 15 min. Make sure that the gel does not run dry.
6. Remove solution A with a 10-mL pipet, cover the gel with solution B, and denature the DNA for 15 min.
7. Remove solution B with a 10-mL pipet, cover the gel with solution C, and neutralize for 15 min.
8. Remove solution C with a 10-mL pipet, cover the gel with 20X SSC, and transfer the DNA onto the nylon membrane by vacuum blotting for 90 min.
9. Remove the 20X SSC and the gel and rinse the nylon membrane in a tray with 0.4 N NaOH for 30 s.
10. Remove the 0.4 N NaOH and rinse the nylon membrane with 0.2 M Tris-HCl, pH 7.5, for 30 s.
11. Place the membrane on a sheet of Whatman paper to remove excess liquid and fix the DNA on the nylon membrane using a UV crosslinker.
12. Rinse the membrane briefly in 5X SSC, put it in a hybridization oven bottle (DNA side should face the interior, not the glass walls), and add 15 mL of ECL hybridization solution prewarmed to 42°C. Prehybridize for 1 h at 42°C.

13. While prehybridizing the membrane, label the gel-purified probe using the ECL kit (see Subheading 3.3.).

14. Pour one-half of the hybridization solution from the hybridization tube into a 12-mL plastic tube. Add the labeled probe, mix briefly, and pour the solution back into the hybridization tube with the membrane. Hybridization is performed overnight at 42°C.

15. Remove the hybridization solution. Add 50 mL 5X SSC to the hybridization bottle and incubate for 5 min in the hybridization oven.

16. Remove the 5X SSC. Add 50 mL of prewarmed washing buffer I and incubate for 20 min in the hybridization oven at 42°C.

17. Repeat the washing step twice for 10 min with new washing buffer I.

18. Put the membrane into a plastic tray and wash twice at room temperature with washing buffer II for 5 min.

19. Put the membrane with the DNA side up into a new plastic tray. Mix 8 mL of detection solution I with 8 mL of detection solution II (both provided in the kit) in a 50-mL plastic tube, pour it carefully on the surface of the membrane (the membrane must be completely covered), and expose for 1 min.

20. Pour the detection solution back into the plastic tube. Place the nylon membrane with the DNA side down onto plastic wrap and fold the ends of the wrap around the membrane.

21. Place the wrapped nylon membrane into a cassette (DNA side up), put an ECL Hyperfilm on it, and expose for 10 min at room temperature in the dark. Develop the film and, if necessary, expose another film for a shorter or longer time, depending on the signal strength. The membrane can also be stored in the used detection solution in a sealed plastic bag for rehybridization with the same or another probe.

3.3. Probe Preparation

1. Amplify the 954 bp CARE-2 fragment in a standard polymerase chain reaction (PCR) using plasmid pRFL37 (3) as template and the primer pair 5’-CTCTAAAACTGTGCTTGGTG-3’ and 5’-AATTTCACACATCGAGAGC-3’ (see Note 1).

2. After completion of the PCR, mix with an appropriate volume of 6X loading buffer and load the sample on a 1% agarose gel. Run the gel overnight at 30 V in 1X TAE buffer. Stain the gel with ethidium bromide and excise the PCR product under UV light using a scalpel. Excise as precisely as possible to remove excess agarose.

3. We use the GeneClean kit from Q-BIOgene to purify the fragment. Place the agarose plug with the CARE-2 fragment into a 1.5-mL Eppendorf cap, add 400 µL (3 volumes the gel slice) sodium iodide solution (provided in the kit), and incubate at 50°C until the agarose is completely dissolved (approx 5 min).
4. Add 8 µL of glass milk (provided in the kit), vortex briefly, and incubate for 5 min on ice.
5. Pellet by centrifugation for 10 s, remove the supernatant, and resuspend the pellet in 200 µL of “New wash” solution (provided in the kit). Centrifuge for 10 s and repeat the washing step two times.
6. Suspend the pellet in 12 µL of double-distilled H₂O and incubate for 5 min at 50°C.
7. Centrifuge at 16,000g for 2 min and transfer the supernatant with the DNA to a new 1.5-mL Eppendorf cap.
8. Control the quality of the eluted fragment by analyzing 1 µL of the sample on an agarose minigel together with a known amount of a size marker. The remainder is kept on ice until use or stored at −20°C.
9. Transfer 100 ng of the CARE-2 fragment to a new 1.5-mL Eppendorf cap and add double-distilled H₂O to 10 µL. Denature the DNA by incubation at 100°C for 10 min, centrifuge briefly, and place the cap immediately on ice to avoid reannealing of the DNA strands.
10. Add 10 µL of labeling reagent (provided in the kit) and vortex briefly. Centrifuge briefly.
11. Add 10 µL of the glutaraldehyde solution, vortex briefly, centrifuge briefly, and incubate at 37°C for 10 min.
12. Directly use the labeled fragment for hybridization (see Subheading 3.2.) or keep on ice until use (up to 15 min).

4. Notes

1. The CARE-2 element is degenerate. Therefore, some hybridizing bands will give a stronger, others a weaker signal. Use pRFL37 described by Lasker et al. (3) as a source of the CARE-2 element instead of amplifying it from genomic DNA of C. albicans to make sure that you always use the same sequence as probe. For optimal comparison of hybridization patterns use the ethidium bromide-stained gel to check that equal amounts of DNA have been loaded for all strains.

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References


Targeted Gene Deletion in *Candida albicans* Wild-Type Strains by *MPA* \(^R\) Flipping

Joachim Morschhäuser, Peter Staib, and Gerwald Köhler

Summary

Many genetic and phenotypic changes occur during the development of drug resistance in fungi. A straightforward approach to assess the contribution of a specific gene to drug resistance is to examine its inactivation in a resistant isolate and to analyze the effect of the mutation on the resistance phenotype. The generation of knockout mutants in the diploid yeast *Candida albicans* requires two rounds of gene replacement to inactivate both alleles of a target gene. Because auxotrophic markers are not useful for the genetic manipulation of wild-type, clinical isolates, dominant selection markers are required. In this chapter, we describe the *MPA* \(^R\) flipping method that combines dominant selection with recombinase-mediated marker recycling for targeted inactivation of specific genes in *C. albicans* wild-type strains. Using the *MPA* \(^R\) flipper makes drug-resistant clinical isolates amenable to genetic manipulation, a prerequisite for the study of causal relationships between specific genes and drug resistance.

**Key Words:** *Candida albicans*; dominant selection marker; drug resistance; efflux pumps; gene disruption; marker recycling; site-specific recombinase.

1. Introduction

*Candida albicans* is a diploid organism without a known haploid phase. To obtain null mutants in which a specific gene is inactivated, both alleles of the gene have to be deleted from the genome by allelic replacement, whereby a disrupted copy of the gene is substituted for the wild-type alleles. To identify mutants carrying the inactivated gene copy, a selection marker is inserted between flanking sequences of the target gene, which serve for specific integration of the marker into the target locus by homologous recombination. Until recently, mutant construction was limited to auxotrophic *C. albicans* laboratory strains with a defect in certain biosyntheses (e.g., uridine, arginine, histidine) that can be complemented using the corresponding intact genes (e.g., *URA3, ARG4, HIS1*) as selection markers. Homozygous mutants are obtained
either by using different markers for inactivation of the two alleles in a host strain with multiple auxotrophies or by repeated use of a counterselectable, recyclable marker (e.g., \textit{URA3}) for sequential disruption of both alleles of the target gene (1). These methods are therefore not applicable to prototrophic wild-type strains, like drug-resistant clinical isolates. However, the contribution of a gene to a certain phenotype, like drug resistance, may not be revealed in laboratory strains. A well-documented example is the \textit{MDR1} gene, which encodes an efflux pump that is overexpressed in many fluconazole-resistant \textit{C. albicans} isolates. Inactivation of \textit{MDR1} in \textit{C. albicans} laboratory strains did not affect the susceptibility of the strains to fluconazole, because these strains express the \textit{MDR1} gene at very low levels under the conditions used (2,3). In contrast, disruption of the gene in \textit{MDR1}-overexpressing clinical isolates (using the method described in this chapter) abolished their resistance, providing direct genetic proof that \textit{MDR1} overexpression mediates fluconazole resistance in \textit{C. albicans} (4).

Genetic manipulation of \textit{C. albicans} wild-type strains is now relatively straightforward because a dominant selection marker, which is based on the \textit{C. albicans IMH3} gene (5), has become available. The \textit{IMH3} gene encodes inosine monophosphate dehydrogenase, a key enzyme in the \textit{de novo} biosynthesis of guanine nucleotides that is inhibited by mycophenolic acid (MPA). \textit{C. albicans} transformants carrying a single copy of a mutated allele of the \textit{IMH3} gene (termed \textit{MPAR}), which encodes an MPA-resistant inosine monophosphate dehydrogenase, can grow on minimal plates containing high concentrations of MPA. Therefore, \textit{C. albicans} transformants carrying the \textit{MPAR} marker inserted at a specific genomic locus can be selected on MPA-containing agar plates on which growth of the wild-type parent strains is inhibited (6,7).

For the construction of homozygous \textit{C. albicans} mutants, however, the \textit{MPAR} marker has to be recycled after insertion into the first allele of the target gene to allow its subsequent use in a second round of transformation for inactivation of the remaining wild-type allele. Because a negative selection against the presence of the \textit{MPAR} marker is not available in \textit{C. albicans} wild-type strains, we developed a method that allowed the specific excision of the marker from the genome of transformants with high efficiency. We designed a cassette, the \textit{MPAR} flipper, which in addition to the \textit{MPAR} marker, contains a \textit{C. albicans}-adapted \textit{FLP} gene (\textit{caFLP}) encoding the site-specific recombinase \textit{FLP}, under the control of the inducible \textit{SAP2} promoter. The cassette is flanked by direct repeats of the minimal recombination target sites (\textit{FRT}) of the \textit{FLP} recombinase (Fig. 1).

After adding flanking sequences of a target gene, the cassette can be used to transform \textit{C. albicans} wild-type strains to MPA resistance (Fig. 1A,B). Transformants containing an integrated cassette in one allele of the target gene
are passaged in a *SAP2* promoter-inducing growth medium. This results in the expression of the FLP recombinase, which in turn recombines its target sites and thereby excises the cassette from the genome. The circularized, excised cassette does not replicate and is lost from the cells. The resulting MPA-sensitive derivatives can be identified by their slower growth on plates containing a low concentration of MPA; they form smaller colonies than the original transformants (Fig. 2C). The MPA-sensitive heterozygous mutants are then used for a second round of integration and subsequent excision of the *MPAR* flipper cassette to obtain the desired homozygous mutants. The *MPAR*-flipping strategy has been successfully used for targeted inactivation of various genes in different *C. albicans* and *Candida dubliniensis* strains (4,8–11).

2. Materials

1. Plasmid pSF11.
3. GeneClean kit (GeneClean III Kit, Q-BIOgene, Heidelberg, Germany).
4. Double-distilled H₂O.
5. 50X TAE buffer: 242 g Tris-HCl, 57.1 mL of acetic acid, and 100 mL of 0.5 M ethylene diamine tetraacetic acid (EDTA), pH 8.0; add H₂O to 1 L.
6. Yeast peptone dextrose (YPD) medium: 2% peptone, 1% yeast extract, 2% glucose.
7. 1 M Lithium acetate, pH 7.5.
8. 10X TE buffer: 100 mM Tris-Cl, pH 7.5, 10 mM EDTA.
9. 1 M Sorbitol.
10. Electroporation cuvettes (Electroporation cuvet 2 mm, Equibio, Kent, UK).
11. Electroporator (Easyject Prima, Equibio).
12. 10 µg/mL MPA-plates: dissolve 6.7 g of yeast nitrogen base (YNB, with ammonium sulfate, without amino acids or dextrose; Q-BIOgene) in 800 mL of double-distilled H₂O, pH adjusted to 5.8 with NaOH. Add 15 g of agar and autoclave. After autoclaving, cool to approx 60°C and add 100 mL of a sterile 20% glucose solution and 100 mL of a sterile 10X stock solution of complete supplement medium (7.7 g/L CSM [Q-BIOgene] in double-distilled H₂O). Add 500 µL of a mycophenolic acid stock solution (20 mg/mL MPA [Sigma-Aldrich, St. Louis, MO] in 100% ethanol). The MPA stock solution is stored at –20°C. The MPA must be thoroughly dissolved by vortexing before use.
13. 1.5 µg/mL MPA-plates (prepare like 10 µg/mL MPA-plates but instead of 500 µL, add only 75 µL of the MPA stock solution per liter).
14. SD plates (prepare like MPA plates but do not add MPA).
15. YCB-bovine serum albumin (BSA) medium: dissolve 23.4 g of yeast carbon base (YCB, Difco, Augsburg, Germany) and 2 g yeast extract in 1 L of double-distilled H₂O, adjust the pH to 4.0 with HCl, and autoclave. Before use, add 400 µL of a sterile-filtered 10% BSA (Fraction V; Gerbu Biotechnik GmbH, Gaiberg, Germany) stock solution.
4. 10X phosphate-buffered saline (PBS): 80 g of NaCl, 2 g of KCl, 14.4 g of Na$_2$HPO$_4 \times 2$ H$_2$O, and 2.4 g of KH$_2$PO$_4$; add H$_2$O to 1 L.

3. Methods

3.1. Construction of the Disruption Cassette

1. The MPA$^R$ flipper cassette in plasmid pSFI1 was constructed in the vector pBluescript II KS and was designed to contain several unique restriction sites on the left (ApaI, XhoI) and right (NotI, SacII, SacI) borders. These sites can be used for cloning flanking sequences of the target gene that serve for specific genomic integration by homologous recombination. For example, an upstream fragment is amplified by polymerase chain reaction (PCR) with primers introducing a distal ApaI site and a proximal XhoI site, and a downstream fragment is similarly obtained with primers introducing a proximal SacII site and a distal SacI site. The fragments are then cloned into the appropriately digested pSFI1 (see Note 1).

2. Excise the complete cassette from the plasmid (in the example given above by digestion with ApaI and SacI) and separate the fragment from the vector by agarose gel electrophoresis. Run the gel overnight on a 1% agarose gel in 1X TAE buffer at 40 V. After ethidium bromide staining, excise the fragment containing the disruption cassette and purify it using the GeneClean kit. Dissolve the DNA in 6 $\mu$L double-distilled H$_2$O.

3. Control the quality of the eluted fragment by analyzing 1 $\mu$L of the sample on an agarose minigel together with a known amount of a size marker. The remaining 5 $\mu$L is used for electroporation and should contain at least 1 $\mu$g of DNA for a successful transformation.

Fig. 1. (opposite page) Construction of homozygous Candida albicans mutants by targeted gene disruption using the MPA$^R$-flipping strategy. The structure of the MPA$^R$ flipper cassette is shown on top. The C. albicans-adapted FLP gene (caFLP, light grey arrow) is placed under the control of the inducible SAP2 promoter (SAP2p, indicated by the angled arrow) and fused to the transcription termination sequence of the ACT1 gene (ACT1t, indicated by the black circle). The MPA$^R$ marker is shown as a dark grey arrow, and the FLP recombination target sites by the short black arrows. The unique flanking restriction sites are: A, ApaI; N, NotI; ScI, SacI; ScII, SacII; and Xh, XhoI. In the example shown, upstream and downstream sequences of the target gene (white boxes, labeled 5' and 3') are cloned as ApaI-XhoI and SacII-SacI fragments, respectively, on both sides of the MPA$^R$ flipper cassette to obtain the disruption construct. After the first round of transformation of the wild-type parental strain, MPA-resistant clones are obtained in which one allele of the target gene open reading frame (indicated by the hatched arrow) is replaced by the MPA$^R$ flipper. FLP-mediated excision of the cassette results in MPA-sensitive derivatives, which are then used for a second round of integration/excision of the MPA$^R$ flipper to obtain homozygous mutants. Apart from the inactivation of the target gene the final mutants are identical to the wild-type parental strain. MPA, mycophenolic acid.
Targeted Gene Deletion in C. albicans

**Fig. 1.**

A \( \times h \) FRT SAP2p caFLP ACT11 \( MPAR \) FRT

\[ N \text{ScI} \text{Scl} \]

**MPAR** flipper cassette
(contained in plasmid pSF11)

Add target gene flanking sequences

Disruption construct

Homologous recombination

Genome of diploid parent strain

Gene replacement

Heterozygous mutant (MPA-resistant)

FLP-mediated excision

Heterozygous mutant (MPA-sensitive)

2nd round of gene replacement

Homozygous mutant (MPA-resistant)

FLP-mediated excision

Homozygous mutant (MPA-sensitive)
Fig. 2. Selection of mycophenolic acid (MPA)-resistant *Candida albicans* transformants and screening for MPA-sensitive derivatives in which the *MPA*<sup>R</sup> flipper cassette was excised by FLP-mediated recombination. (A) A *C. albicans* wild-type strain was transformed with a deletion construct containing the *MPA*<sup>R</sup> flipper cassette inserted between flanking sequences of a target gene. The photograph was taken after 7 d of growth of the transformants at 30°C on an synthetic defined (SD) agar plate containing 10 µg/mL MPA. (B) Several small colonies (clones 1–3) were picked, restreaked on an SD agar plate containing 10 µg/mL MPA, and incubated for 3 d at 30°C. The MPA-sensitive wild-type parental strain was included as a negative control. (C) Appearance of large MPA-resistant colonies and small MPA-sensitive derivatives (two are marked by arrows) which, after induction of *FLP* expression, were grown for 3 d at 30°C on an SD agar plate containing 1.5 µg/mL MPA.
3.2. Transformation of C. albicans by Electroporation

1. Prepare an overnight culture of your C. albicans host strain in YPD medium.
2. Inoculate an Erlenmeyer flask containing 50 mL of YPD medium with 5 µL of the preculture and grow overnight at 30°C on a rotary shaker set to 250 rpm.
3. When the culture has reached an optical density (OD)_{600nm} of 1.6–2.2, transfer the cells into a sterile 50-mL tube and centrifuge for 5 min at 3300g.
4. Carefully remove the supernatant using a 10-mL glass pipet and resuspend the cells in 8 mL of sterile double-distilled H₂O.
5. Add 1 mL of 10X TE buffer, pH 7.5, and mix.
6. Add 1 mL of 1 M lithium acetate, pH 7.5, and mix.
7. Incubate at 30°C in a rotary shaker set to 150 rpm for 60 min.
8. Add 250 µL of 1 M dithiothreitol and incubate in a rotary shaker for another 30 min at 30°C.
9. Add 40 mL of sterile, cold double-distilled H₂O, mix, and centrifuge the cells for 5 min at 3300g, 4°C.
10. Place the tube on ice, remove the supernatant using a 10-mL glass pipet, resuspend the cells in 25 mL sterile, cold, double-distilled H₂O, and centrifuge the cells for 5 min at 3300g, 4°C.
11. Resuspend the cells on ice in 5 mL of sterile, cold 1 M sorbitol and centrifuge for 5 min at 3300g, 4°C.
12. Resuspend the cells on ice in 50 µL of sterile, cold 1 M sorbitol (this should result in a dense suspension of a total volume of about 150 µL). Divide the cell suspension in 40-µL aliquots in sterile Eppendorf tubes and keep on ice.
13. Mix the 5 µL of the gel-purified DNA fragment containing the disruption cassette with 40 µL of electrocompetent cells. Transfer to a precooled electroporation cuvette on ice. Include a mock electroporation without DNA.
14. Place the electroporation cuvet into the Equibio electroporator. Electroporate the cells at 1.8 kV and place the cuvet back on ice.
15. Add 1 mL of sterile, cold 1 M sorbitol to the cells, mix using a sterile Pasteur pipet, and transfer the cell suspension into a 1.5-mL Eppendorf cap.
16. Plate 100 µL of the suspension on an synthetic defined (SD) agar plate containing 10 µg/mL MPA. In addition, prepare a dilution series and plate 100 µL of a 10⁻⁵ and a 10⁻⁶ dilution on a control plate (without MPA) to determine the number of viable cells.
17. Pellet the remaining cell suspension by centrifugation at 3300g for 2 min, remove the supernatant, and resuspend the cells in 100 µL of 1 M sorbitol. Plate the cell suspension on an SD agar plate containing 10 µg/mL MPA (this plate can usually be discarded because enough transformants are normally obtained from the first plate with only 100 µL of the cell suspension plated, the latter having less background growth of untransformed cells).
18. Incubate the control plate for 2 d at 30°C and count the colonies to determine the number of viable cells in the original cell suspension after the electroporation.
19. Incubate the selection plates for 5–7 d at 30°C. Count the MPA-resistant colonies emerging from the thin background lawn of nontransformed cells and determine the transformation frequency (see Note 2).

20. Pick 12 MPA-resistant colonies with a sterile toothpick and restreak them for single colonies on a fresh SD agar plate with 10 µg/mL MPA. Incubate for 3 d at 30°C, pick an individual colony of each transformant, streak on an SD plate without MPA, and grow for 2 d at 30°C. Thereafter, the transformants are maintained without further selection pressure.

21. The clones are then analyzed for the desired allelic replacement by sequential Southern hybridizations (see Chapter 3) using the upstream and downstream flanking fragments of the disruption cassette as probes (see Notes 2 and 3).

3.3. Excision of the MPA^R Flipper Cassette

1. Pick a single colony of a clone containing the MPA^R flipper cassette in one allele of the target gene from an SD agar plate. Suspend the cells in an Erlenmeyer flask containing 10 mL of YCB–BSA medium and grow them for 18 h (overnight) at 30°C in a rotary shaker set to 250 rpm. This should yield a dense culture.

2. Prepare a dilution series in 1X PBS and plate 100 µL of the 10^-5 and 10^-6 dilutions onto an SD agar plate containing 1.5 µg/mL MPA. Ideally, there should be between 50 and 200 colony forming units on the plates.

3. Incubate the plates for 3 d at 30°C. Pick several small colonies and restreak them onto SD agar plates and also onto SD agar plates containing 10 µg/mL MPA to confirm that the clones are MPA-sensitive (there should be no growth at the high MPA concentration, as in the original wild-type parent).

4. Pick a single colony of several MPA-sensitive clones and verify deletion of the MPA^R flipper cassette by Southern hybridization analysis. One correct MPA-sensitive derivative is then used for a second round of integration/excision of the MPA^R flipper to obtain homozygous mutants.

4. Notes

1. Use long flanking sequences (approx 0.5–1 kb) for the disruption construct. This increases the probability that the cassette will be integrated into the target locus. Confirm by sequencing that no undesired mutations were introduced by the PCR, which might affect neighboring genes.

2. MPA-resistant transformants may appear already after approx 3 d of growth on the selection plates. In our experience these are usually not the desired integrants. Instead, the MPA^R marker alone seems to have integrated into one of the IMH3 alleles (the transformants look like the wild-type parent in Southern hybridization analyses), and these undesired transformants apparently grow somewhat faster than clones in which the complete cassette is integrated into the target locus. It is therefore wise to wait for 5–7 d before picking transformants. Nevertheless, the correct integrants may still be a minority, and one should test at least 12 transformants by Southern hybridization in a first analysis. In our hands the frequency of correct integrants varied between 2 and 50% when using the MPA^R flipping method in C. albicans, depending on the target locus and the length of...
the flanking sequences, or the quality of the DNA fragment used for transformation. In cases in which the specific integration is rare, the transformants may first be screened by PCR using a primer binding within the MPAR flipper cassette and a primer that binds in the target locus outside of the cloned flanking sequences. However, Southern hybridization analysis must always be the final confirmation, because PCR does not detect additional, ectopic integrations (this also applies to the use of other markers and procedures for integrative transformation of C. albicans). If no correct clones are obtained after screening of a reasonable number of MPA-resistant transformants (approx 20–30 clones) it is better to repeat the transformation with a freshly prepared disruption cassette.

3. Transformation of C. albicans can cause undesired genomic alterations. To make sure that phenotypes are the result of inactivation of the target gene, construct at least two independent homozygous mutants, starting from two independent first round transformants. In addition, an intact copy of the target gene should be reintegrated into the homozygous mutants, and this should revert the mutant phenotype. Reintroduction can be achieved by substituting a complete copy of the gene, including upstream and downstream sequences, for the upstream region in the deletion construct. After integration into one of the inactivated alleles the MPAR flipper cassette is excised again to remove the selection marker.

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References


Application of Deoxyribonucleic Acid Microarray Analysis to the Study of Azole Antifungal Resistance in Candida albicans

Katherine S. Barker and P. David Rogers

Summary

The near completion of sequencing the Candida albicans genome has made it possible to employ genomic technologies, such as microarray analysis, to aid in identifying key genes involved in such clinical problems as the acquisition of high-level resistance to azole antifungal agents. Here, we outline in detail the methodologies utilized in our laboratory to culture clinical isolates of C. albicans, isolate ribonucleic acid from such cultures, synthesize labeled complimentary deoxyribonucleic acid probes from the ribonucleic acid samples, hybridize the probes to microarray chips, analyze the data from such hybridizations, and validate results using reverse transcriptase-polymerase chain reactions. Microarray analysis gives researchers the ability to identify genes involved in processes such as acquisition of azole resistance and to use the data in a way that may lead to clinical approaches to inactivate these genes and improve patient outcomes.

Key Words: Candida albicans; DNA microarray; RNA; RT-PCR; azole resistance.

1. Introduction

Azole antifungal agents have proven effective for the management of Candida albicans infections such as oropharyngeal candidiasis, the most frequent opportunistic infection among patients with acquired immunodeficiency syndrome (1,2). However, the repetition and lengthy duration of therapy for oropharyngeal candidiasis in this patient population has led to an increased incidence of treatment failures secondary to the emergence of azole resistance in this pathogenic fungus (3–6). Studies designed to determine mechanisms of azole resistance in C. albicans have uncovered a handful of genes involved in acquisition of high-level resistance: ERG11, CDR1 and CDR2, and MDR1 (7–16). Until recently, molecular techniques available to examine differential gene
expression (between genetically related or “matched” susceptible and resistant *C. albicans* isolates, for example) have been limited to Northern hybridization, RNase protection assays, reverse-transcription polymerase chain reaction (RT-PCR), subtractive hybridization, and differential display. Northern hybridization, RNase protection assays, and RT-PCR are similar in that these techniques can only compare differential expression of one with a handful of genes based on the input of known sequence in the system. For example, Northern hybridization of a membrane containing RNA samples from susceptible and resistant matched isolates of *C. albicans* can be successfully hybridized simultaneously with probes from several genes provided that the sequences of these genes are known and transcript sizes of the genes hybridized are sufficiently different to afford adequate resolution between them.

These techniques, therefore, are not designed to find genes never before identified as being differentially expressed. However, differential display and subtractive hybridization are techniques used to identify the differential expression of potentially novel genes. However, they are slow processes restricted by the sets of primers chosen to be used during amplification steps (differential display) or by the number of clones picked for sequencing after subtraction (subtractive hybridization).

The comparatively newer technique of microarray analysis allows for the simultaneous examination of hundreds or thousands of genes, according to the number of genes whose sequences are known in a given genome. With the sequencing of both the *Saccharomyces cerevisiae*, and more recently the *C. albicans* genomes, the use of functional genomics has been increasingly adaptable to the study of azole antifungal resistance (17–21). This chapter describes the application of deoxyribonucleic acid (DNA) microarray analysis for the identification of genes that are differentially expressed in association with the azole-resistant phenotype.

2. **Materials**

2.1. *C. albicans* **Cell Culture and RNA Isolation**

1. Yeast peptone dextrose (YPD) broth: 1% yeast extract, 2% peptone, 1% dextrose.
2. Glycerol.
3. Cuvets suitable for measuring optical density in ultraviolet (UV) spectrum.
4. 250-mL Polypropylene centrifuge bottles.
5. AE buffer: 50 mM sodium acetate (pH 5.2), 10 mM ethylene diamine tetraacetic acid (EDTA).
6. Phenol-resistant, polypropylene copolymer Oak Ridge tubes.
7. 20% Sodium dodecyl sulfate (SDS).
8. Saturated phenol, pH 4.3.
2.2. Probe Preparation and Microarray Hybridization

1. Deoxynucleotides.
2. T20VN primer.
3. Tris-HCl.
4. Dithiothreitol.
5. Potassium chloride.
6. 1 M Magnesium chloride solution.
7. RNasin.
8. Superscript II reverse transcriptase.
9. 0.5 mL of RNase-free microcentrifuge tubes.
10. Sodium hydroxide.
11. Acetic acid.
12. Molecular-grade (nuclease-free) water.
13. QIA-quick nucleotide removal kit (containing Buffer PB, Buffer PE, and purification columns).
15. Cy3-labeled deoxycytidine 5’-triphosphate (dCTP).
16. Cy5-labeled dCTP.
17. *C. albicans* microarray chips.
18. Salmon sperm DNA.
20. Hybridization chamber.
21. 20X SSC: 3 M NaCl, 0.3 M sodium citrate.
22. Microarray scanner.

2.3. cDNA Synthesis and RT-PCR

1. Oligo(dT) primer or random hexamer primers.
2. RNase block ribonuclease inhibitor.
4. SuperScript™ First-Stand Synthesis System for RT-PCR (containing RT reaction buffer).
5. Heat block with temperature range between 37 and 100°C.
6. PCR primers.
8. EasyStart Micro 50 tubes (provided with 0.1% Triton X-100 solution).
3. Methods

DNA microarray analysis allows for the measurement of steady-state messenger ribonucleic acid (mRNA) levels for most genes in the *C. albicans* genome under a given condition. Total RNA is isolated from cells under the conditions to be compared and reverse transcribed into Cy3- or Cy5-labeled cDNA. The two cDNA samples are then hybridized to the same DNA microarray. The fluorescent signals for both dyes are imaged and normalized. The ratios between the two signals for each gene give an indication of relative gene expression. DNA microarray analysis is well suited for the assessment of differential gene expression between isolates within matched sets of azole-susceptible and -resistant *C. albicans*. Although we routinely perform experiments in replicate at minimum, we also confirm the differential expression of select genes independently using RT-PCR. Other methods, such as Northern blot or real-time RT-PCR, are also appropriate.

3.1. *C. albicans* Growth Conditions

1. Dilute an aliquot of glycerol stock (see Note 1) from each isolate in YPD broth and grow overnight at 30°C in an environmental shaking incubator. This step will produce a dense, overgrown culture.
2. Dilute precultures overnight to an optical density of 600 nm (OD$_{600}$) of 0.2 in fresh YPD and grow as before to early logarithmic phase at an equivalent OD (0.4–0.5) for subsequent RNA isolation.

3.2. RNA Isolation

Our preferred method for isolation of total RNA from *C. albicans* is the hot phenol method (22). This method yields up to 2 mg of total RNA from cultures of 500 mL.

1. Collect cells by centrifugation at 500g, discard supernatants, snap-freeze cell pellets in liquid nitrogen, and store at −70°C until ready to continue the protocol. Cell pellets can be stored in this fashion for up to 3 mo.
2. Resuspend frozen cells in 12 mL of AE buffer at room temperature and transfer to phenol-resistant Oak Ridge tubes, followed by the addition of 800 µL of 20% SDS and 12 mL of acid phenol (see Note 2).
3. Incubate the cell lysate for 10 min at 65°C with vortexing each minute, cool on ice for 5 min, and subject to centrifugation for 15 min at 11,952g in a Sorvall RC-5B (or similar high-speed) centrifuge. The hot phenol incubation solubilizes the cell wall while the centrifugation step separates the cell lysate into aqueous and organic supernatant phases and a pellet containing cellular debris.
4. Transfer both phases of supernatant to new Oak Ridge tubes containing 15 mL of chloroform. Mix and subject to centrifugation at 200g for 10 min.
5. Transfer aqueous layer (see Note 3) to new tubes containing 1 volume isopropanol (approx 14 mL) and 0.1 volume of 2 M sodium acetate, pH 5.0. Mix well and subject the mixture to centrifugation at 17,211g for 35 min at 4°C.
6. Remove the supernatants, resuspend the pellet in 10 mL of 70% ethanol, and collect the RNA by centrifugation at 17,211g for 20 min at 4°C.
7. Remove supernatants completely and dissolve RNA in DEPC-treated water. A volume of 100 µL of water will easily achieve a concentration of greater than 2 µg/µL RNA necessary for probe preparation (see Note 4).
8. Obtain the ODs at 260 and 280 nm spectrophotometrically by measuring 5 µL of each RNA sample diluted with 495 µL of TE, and the integrity of the RNA is visualized by subjecting 2 µL of the sample to electrophoresis through a 1% agarose–1X MOPS buffer gel.

3.3. Microarray Design and Preparation

The C. albicans microarray currently used by our laboratory is manufactured by Eurogentec SA (Ivoz-Ramet, Belgium) in collaboration with the European Galar Fungail Consortium (www.pasteur.fr/recherche/unites/Galar_Fungail/). Primers for each of the 6039 putative open reading frames (ORFs) in the C. albicans genome were designed by Eurogentec SA to amplify a specific region of each ORF. Both the forward and reverse primers for each ORF contained a 15-bp universal sequence at the 5' end to generate 5' amino-modified product for covalent attachment to aldehyde-coated glass slides. Amplicons were an average length of 300 bp and were spotted in duplicate, along with 27 control genes, using a ChipWriter Pro (Virtek Vision Intl., Waterloo, Ontario, Canada) robotic array printer.

3.4. Probe Preparation (see Note 5)

1. Add 10 µg of total RNA sample, at a concentration greater than or equal to 2 µg/µL, to a mixture of 1 pmol of T20VN and oligo(dT) (18- to 21-mer) primer mix; 0.5 mM (each) deoxyadenosine 5'-triphosphate, deoxyguanosine 5'-triphosphate, and deoxythymidine 5'-triphosphate; 20.5 µM dCTP; 37.5 µM Cy3- or Cy5-dCTP; and 10 mM dithiothreitol in a buffer containing 50 mM Tris-HCl, pH 8.3; 75 mM KCl; and 3 mM MgCl₂. Take care to use the correct label (Cy3 or Cy5) for each sample being competitively hybridized.
2. Denature the reaction mixture at 65°C for 5 min and incubate at 42°C for 5 min, followed by the addition 1 µL of RNasin and 200 U of Superscript II reverse transcriptase.
3. Incubate the reaction mixture at 42°C for 1 h, then add 200 U of Superscript II reverse transcriptase, and incubate at 42°C for an additional hour.
4. Stop the reaction by adding EDTA (pH 8.0) and sodium hydroxide to a final concentration of 5 mM and 0.4 N, respectively, and incubate the mixture at 65°C for 20 min. Finally, add acetic acid to achieve a final concentration of 0.37 M.
5. To purify the labeled cDNA probes, adjust the volume of the cDNA reaction to 100 µL with water and mix with 0.5 mL of buffer PB (provided in the Qiagen nucleotide removal kit) and 13 µL of 3 M sodium acetate (pH 5.2). This and the remaining steps serve to remove and free nucleotides from the labeling reaction and to concentrate the complementary DNA probe to an optimal volume for hybridization.

6. Apply the sample to a QIA-quick column (provided in the Qiagen nucleotide removal kit) and centrifuge at 17,982 g in a microcentrifuge for 60 s.

7. Discard the flowthrough, and wash column twice with 750 µL of buffer PE with a 60-s centrifugation after each wash.

8. After an additional centrifugation step to dry the column, apply 50 µL of prewarmed (42°C) water to the column and incubate for 3 min at room temperature.

9. To elute the sample, centrifuge the column as before, and then perform an additional elution step.

10. Pool eluates, apply to a Microcon-30 filter, and centrifuge as before for three minutes. Vortex the membrane unit briefly and centrifuge for an additional 60 s. Adjust the final eluate volume to 5 µL with water.

3.5. Microarray Hybridization

1. Mix 5 µL each of the Cy3- and Cy5-labeled probes with 50 µg of heat-denatured salmon sperm DNA, incubate at 95°C for 2 min, and snap-cool on ice.

2. Add the mixture to 40 µL of hybridization buffer and apply to the array slides under glass cover slips.

3. Perform the hybridization at 37°C overnight in a humidified hybridization chamber.

4. To wash the slides, remove the cover slip, and incubate the slide at room temperature in 0.2X SSC plus 0.1% SDS for 5 min with agitation. Rinse at room temperature with 0.2X SSC for five minutes with agitation, and spin-dry at approx 100 g for 5 min.

5. Slides are scanned using a ChipReader microarray scanner (Virtek Vision International; see Note 6).

3.6. Data Analysis

1. Use microarray image analysis software, such as GenePix 1.0 software (Axon Instruments, Inc., Union City, CA), image analysis, and data visualization. Calculate the local background values from the area surrounding each spot and subtract from the total spot signal values. These adjusted values are used to determine differential gene expression (Cy3/Cy5 ratio) for each spot. An image of a hybridized C. albicans microarray is shown in Fig. 1.

2. Apply a normalization factor to account for systematic differences in the probe labels by using the differential gene expression ratio to balance the Cy5 signals.

3. Eurogentec provides gene annotation (gene name and gene function) for each gene spotted on the array. Further annotation on characterized genes may be per-
Fig. 1. This is a representative microarray from a competitive hybridization of labeled probes from a matched pair of susceptible and resistant *Candida albicans* isolates. Each dot is *C. albicans* DNA spotted onto the glass slide and hybridized with the mixture of both labeled probes.
formed using the results of BLASTn searches from the Stanford University (Palo Alto, Calif.) Candida sequencing database (http://www-sequence.stanford.edu/group/candida), and the CandidaDB database (http://www.pasteur.fr/Galar_Fungail/CandidaDB/).

4. Data may be further analyzed using various software packages, such as Cluster (23) and TreeView (M.B. Eisen laboratory, Stanford University), or GeneSpring (Silicon Genetics, Redwood City, CA). Cluster and TreeView are available as free software programs online. GeneSpring is one of several data analysis systems designed for powerful analysis of any size set(s) of microarray data. Data expressed in a cluster figure generated by Cluster and TreeView are shown in Fig. 2A.

3.7. cDNA Synthesis and RT-PCR

We routinely verify differential expression of selected genes of interest among those identified by microarray analysis (Fig. 2B; see Note 7).

1. To synthesize cDNA, place approx 2 µg of total RNA in a 0.5-mL reaction tube with 1 µg of oligo(dT) primer stock and adjust the volume to 15.5 µL using DEPC-treated water. Alternatively, random hexamers can be used to prime for cDNA synthesis. Typically, using 1 µL of a 50 ng/µL solution of random hexamers (a component of the SuperScript First-stand Synthesis System for RT-PCR; Invitrogen) per 1 µg RNA sample will yield a sufficient quantity of cDNA for these experiments.

2. Incubate the mixture ten minutes at 70°C and chill on ice one minute, then add to the reaction tube the remainder of the reaction mixture from a master mix in order for each reaction to contain a 1.25 mM concentration (each) deoxyadenosine 5’-triphosphate, dCTP, deoxyguanosine 5’-triphosphate, and deoxycytidine 5’-triphosphate; 40 U of RNase inhibitor; and 25 U of Moloney murine leukemia virus reverse transcriptase in RT reaction buffer.

3. After brief mixing, incubate the reaction for 10 min at room temperature, followed by incubation at 37°C for 1 h.

4. Finally, heat the reaction mixture at 90°C for 5 min and either cool on ice for 10 min or immediately store at 4°C until use.

5. PCR is performed by mixing 1 µL of the appropriate dilution of cDNA (empirically determined for each gene to give product in the linear range), 0.5 µg each of forward and reverse primer, 2.5 U of Taq polymerase, and 0.1% Triton X-100 in EasyStart Micro50 PCR tubes and subjecting the reaction mixture to the following reaction conditions: one repetition of 94°C for 5 min; 32 repetitions of 94°C for 1 min, gene-specific annealing temperature for 1 min, and 72°C for 2 min; and 1 repetition of 72°C for 5 min.

6. Equivalent volumes of PCR product are applied to a 3% agarose gel and separated by gel electrophoresis in 1X TAE.
Fig. 2. (A) Cluster images showing genes differentially expressed in this series of isolates. Red represents genes that are upregulated and green represents genes that are down-regulated. Genes shown in both cluster images appear in the same order. Genes are grouped as being coordinately differentially expressed with (I) CaMDR1, (II) CDR genes, (III) both CaMDR1 and CDR genes, or (IV) neither CaMDR1 nor CDR genes. (B) Confirmation of differential expression by reverse-transcription polymerase chain reaction (RT-PCR) of select genes found to be differentially expressed by complementary DNA microarray analysis. RT-PCR of Candida albicans 18S ribosomal RNA was performed as a control.
**4. Notes**

1. *C. albicans* isolates are stored in our laboratory as glycerol stocks (1:1 mixtures of overnight *C. albicans* culture and 40% glycerol) at –70°C.
2. Take proper precautions when handling phenol by wearing protective clothing (laboratory coat, gloves, goggles) and providing proper ventilation by performing the phenol-handling steps in a chemical fume hood.
3. It is important to remove the aqueous phase without disturbing the interphase or organic phase. Therefore, it is better to leave some of the aqueous layer in the tube than to risk carrying over protein or organic contaminants into the isopropanol precipitation.
4. Completely dissolving the RNA pellet in such a small volume of water may take some time. To ensure that RNA integrity is maintained during this step, it is important to keep the RNA on ice as much as possible.
5. Always handle RNA samples with gloved hands.
6. It is preferred to perform at least two hybridizations for each pair of samples to be hybridized. Our laboratory grows two independent sets of cultures for RNA isolation with one set used in one hybridization and the other set used with the Cy3 and Cy5 dyes switched for the other hybridization.
7. For the verification of differential gene expression by RT-PCR, our laboratory uses RNA samples not used in the microarray hybridization in order that we are not merely replicating the microarray results but actually demonstrating true differences in mRNA expression between samples.

**References**


Application of Proteomic Analysis to the Study of Azole Antifungal Resistance in Candida albicans

Massoumeh Z. Hooshdaran, George M. Hilliard, and P. David Rogers

Summary

The sequencing of the Candida albicans genome and recent refinements in protein resolution and identification techniques have greatly enhanced the application of proteomics for the study of this fungal pathogen. Proteome analysis includes the separation and isolation of proteins by two-dimensional polyacrylamide gel electrophoresis and subsequent protein identification by peptide mass fingerprinting using matrix-assisted laser desorption ionization time-of-flight mass spectrometry. This technique has been used for the study of the proteomes of Candida species in the context of virulence, drug response, and resistance. We describe here the application of this approach to the study of azole antifungal resistance in C. albicans.

Key Words: Candida albicans; protein; proteomics; 2D-PAGE; azole antifungal resistance.

1. Introduction

The postgenomic era has allowed for new techniques in protein identification, bringing increased power and utility to proteomic approaches for the study of fungal pathogens. Proteome analysis entails the separation and isolation of proteins by two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) and subsequent protein identification by peptide mass fingerprinting using matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS). This technique has been used for the study of the proteomes of Candida species in the context of virulence, drug response, and resistance (1–6). This approach has been the subject of several reviews in general (7) and specifically for the study of Candida albicans (8–10). Overexpression of several genes and respective gene products have been implicated in azole resistance in this organism, including Erg11p, Cdr1p, Cdr2p, and Mdr1p (11–13). This chapter demonstrates the use of this approach
for the study of azole antifungal resistance and underscores its complementary utility with genomic approaches such as those outlined in Chapter 5.

2. Materials

2.1. Buffers and Reagents

1. Yeast peptone dextrose (YPD) broth (1% yeast extract, 2% peptone, 1% dextrose).
2. Normal saline.
3. Deionized distilled H$_2$O.
4. Ethylene diamine tetraacetic acid (EDTA).
5. Phenylmethylsulfonyl fluoride (PMSF).
6. Complete™ mini (Roche, Mannheim, Germany).
7. Glass beads, 425–600 µ (Sigma Chemical Co., St. Louis, MO).
8. Bead beater.
9. Ice.
10. Coomassie Plus reagent (Pierce Biotechnology, Rockford, IL).
11. 7 M Urea, 2 M thiourea, 2% CHAPS.
12. 0.8% ampholyte, pH 3.0–10.0.
15. Ceramic strip holder.
16. Tweezers.
17. IPG cover fluid.
18. Molecular weight (MW) marker (Rainbow standard marker RPN 800; MW 10,000–250,000; Amersham Biosciences).
19. 24-cm Whatman filter paper.
20. Agarose.
21. 40% Acrylamide/bis solution, 29:1 OR 30% acrylamide/bis, 29:1.
22. 1.5 M Tris-HCl, pH 8.8.
23. Ammoniumpersulphate 10% in water (prepare fresh).
24. Coomassie Blue R-250 (filtered) (Bio-Rad Laboratories, Hercules, CA).

2.2. Equipment

1. Centrifuge (max 6000g).
2. Ultracentrifuge.
3. Refrigerated microcentrifuge.
4. Apparatus for isoelectric focusing and 2D-sodium dodecyl sulfate (SDS) PAGE.
5. Spectrophotometer.
7. Flat rotating shaker.
3. Methods

The methods described below outline the following:

1. Protein isolation from *C. albicans* cultures.
2. Separation and visualization of whole cell protein extract by 2D-SDS PAGE.
3. Excision and subsequent identification of protein spots of interest.

3.1. *C. albicans* Growth Conditions

1. An aliquot of glycerol stock from each isolate is diluted in YPD broth and grown overnight at 30°C in an environmental shaking incubator.
2. Cultures are diluted to an optical density at 600 nM (OD$_{600}$) of 0.2 in 500 mL YPD broth and grown as before for 4.5 h.
3. Cells are collected by centrifugation at 3000 g for 10 min.
4. Cell pellets are washed one time with normal saline and used for further steps (or kept frozen at –80°C until needed).

3.2. Sample Preparation

Proper sample preparation is essential for 2D-gel results when using various types of samples and employing different methods. The optimum procedure must be determined for each sample type to result in a complete solubilization, disaggregation, denaturation, and reduction of the proteins in the sample. Sample preparation depends on the researcher’s final goal. It is advantageous to use as much protein as possible or to follow a reproducible pattern, because the addition of steps used for preparing the sample results in more protein lost per step.

To characterize a protein, it should be rendered completely soluble under conditions of electrophoresis. Specific characteristics of certain proteins will indicate the use of different treatment for solubilizing each one. For example, they may be coupled with membranes, nucleic acid, and other proteins. The effectiveness of solubilization will depend on the method of cell disruption, protein concentration, dissolution, detergent, etc. If any step is not optimized, the sample disruption will not be complete and the 2D-gel results will be affected. For that reason, the choice of dilutant is very important, as is the composition of solution, for 2D-gel results, to avoid unwanted effects on protein PI. The method of cell disruption also is very important; the choice depends on the type of cells—sometimes the lysis of cells should be very gentle; other times, very vigorous lysis is indicated. When disrupting cells, proteases will be released. Therefore, protease inhibitors should be added to protect proteins.

1. Before breaking, cells are washed three times with deionized distilled H$_2$O (*see Note 1*).
2. 500 μL of 50 mM Tris-HCl (pH 7.4) is added to each sample.
3. Protease inhibitors are added to each sample (10 µL of PMSF, 20 µL of EDTA, and 40 µL of Complete mini per 500 µL of sample).
4. Add 0.3 g of glass beads, 425–600 µ to each sample (see Note 2).
5. Cells are next subjected to a bead beater, where they are broken for 30 s and incubated for one minute in ice. This procedure is repeated for a total of five times.
6. Centrifuge for 45 min at 11,250g, 4°C, in a microcentrifuge.
7. Remove the supernatant gently, without disturbing the pellet. At this stage, the pellet consists mainly of unbroken cells, cellular debris, nuclei, and glass beads. Repeat centrifugation as in step 6 for 30 min.
8. Subject the supernatant to ultracentrifugation for 30 min at 445,000g.

### 3.3. Protein Quantification

1. The protein content of the desalted and concentrated sample is measured by the Pierce-modified Bradford method, using bovine serum albumin as a standard.
2. 10 µL of each sample (1:150 v/v) is diluted in water using water as a blank.
3. A replicate (n = 2) aliquot (0.5 mL) of diluted sample, blank, and known concentrations of the standard are also measured.
4. Coomassie Plus reagent (0.5 mL) is added to each tube and mixed, and the absorbance (595 nm) of the solution was measured by spectrophotometry.
5. The results of the standard are used to plot a calibration curve, from which the protein concentration is obtained.
6. Obtain an appropriate volume from each sample, equal to 100-µg protein. In order to have a well-focusing first dimension separation, the sample must be completely disaggregated and fully solubilized.
7. Use the appropriate rehydration solution for the pH range used; for example, 3.0–10.0 NL, which may be stored in 1-mL aliquots in a freezer.
8. Immediately before use, add 0.0093 g of dithiothreitol (DTT)/1 mL volume. Allow it to dissolve for 10 min.
9. In a total volume of 350 µL per IPG strip, combine sample and rehydration solution. Allow to solubilize for 1 h.
10. Centrifuge for 5 min at 10,600g at room temperature.

### 3.4. 2D-SDS PAGE

#### 3.4.1. Isoelectric Focusing (First Dimension)

The first-dimension isoelectric focusing (IEF) procedure has three steps: IPG strip rehydration, sample application, and isoelectric focusing. The IEF method separates proteins based on their isoelectric point (pI). The pI is a specific pH at which the net charge of the protein is zero. Proteins are amphoteric and have either positive, negative, or zero net charge, depending on the pH of their environment. A protein is positively charged in solution with pH below its pI and negatively charges in solution above its pI. Proteins with a negative
change migrate toward the anode and become less negative until their change reaches zero, whereas proteins with positive change migrate toward the cathode, becoming less positively unchanged until they reach zero. This focusing effect of IEF separates proteins close to their pI.

Ready-made IPG strips are available commercially with different pH gradients and different strip lengths. The original method used for first-dimension IEF depended upon the carrier ampholyte-generated pH gradient in the polyacrylamide tube gel. The result is a continuous pH gradient. Because of the limitations of pH gradient formation, immobilized pH gradient or IPG has been developed as an alternative technique for this procedure. The pH 3.0–10.0 NL IPG strips generally have a sigmoidal gradient that gives a better resolution between pH 5.0 and pH 7.0 and shows the widest range of proteins on a single 2-D gel.

Specific steps in the first-dimension IEF procedure are outlined here:

1. Place the IPG strips, on a glass plate that has been cleaned with ethanol, on the bench top and let them thaw 2–3 min. Record the sample name on both sides of the strip.
2. Centrifuge the sample and rehydration buffer for a few minutes to pull down impurities (the sample and rehydration buffer for an 18-cm strip should be approx 350 µL).
3. At this point, two alternatives are equally effective:
   a. Include the sample with the rehydration buffer solution.
   b. Only add rehydration buffer solution to the strip to rehydrate it, then add the sample (see Note 3).
4. Immediately add supernatant to the dry ceramic strip holder. Load at the end point of the strip holder (the end that fits into the positive side of the equipment). Try not to generate any bubbles; if bubbles form, pop them with tweezers.
5. Remove the plastic cover from the square end of IPG strip with tweezers.
6. Place the strip gel-side down on the strip holder, wetting it with sample (move it down slowly, wetting as it moves, preventing the strip from touching a dry ceramic surface). Check for bubbles.
7. Position the strip holder onto the IPGphor gold platform so that the electrode is in the gold section. In the IPGphor IEF system, both rehydration of the IPG strip and IEF occur in the individual strip holder.
8. Pipet 3–4 mL of IPG cover fluid to completely cover the strip holder (oil serves to minimize evaporation and urea crystallization).
9. Place the plastic strip holder cover on top in such a way as to avoid making bubbles.
10. Program the IPGphor based on the sample and rehydration solution composition, on the length of the strip, and on the pH gradient. The following program is used by our laboratory: 3.0–10.0 NL, 18-cm strip (approx 50 µA/strip). A typical IEF protocol generally goes through a series of voltage steps, which begin at low
value (to minimized sample aggregation) and gradually increase to the final desired focusing voltage. Large quantities of protein require more time for focusing. The protocol shown in Table 1 is suitable for first-dimension IEF of a protein sample with IPG buffer concentrations of 0.5–2% in the rehydration solution with 100 µg of protein concentration.

11. Set the program for the appropriate number of strips, at a platform temperature of 20°C.

12. As IEF proceeds, note that the bromophenol blue tracking dye migrates toward the anode.

13. After the program is completed, remove the plastic strip holder cover. Grasp the strip with tweezers and try to drain away the oil on a filter paper.

14. Place the strips right-side up on a strip tray; replace the lid to the tray and leave it at –80°C until electrophoresis occurs.

### 3.4.2. SDS-PAGE

After IEF is complete, the second-dimension separation can be performed on a flat or vertical system based on its MW. In this section, the protein charge is not an issue for separation because of the presence of SDS in the sample and gel. SDS is a detergent and denatures proteins. It also breaks hydrogen bonds and causes proteins to unfold. When proteins are treated with both SDS and a reducing agent such as DTT, separation will occur based on the MW. The larger the polypeptide, the more slowly it migrates in a gel. MW can be determined by migration of protein spots with a standard.

SDS-PAGE consists of the following four steps:

1. Preparation of the second-dimension gel and running buffer, which is a Tris–glycine compound.
2. Equilibrating the IPG strip(s) in SDS buffer.
3. Placing the equilibrated strip on the SDS gel.
4. Electrophoresis.

The percentage of acrylamide usually determines the pore size of the gel, which should be optimized for the sample. The percentage chosen for this study is a homogeneous gel containing 12% total acrylamide. The gel must be ready for use before IPG strip equilibration.

When preparing the stock solution of equilibration buffer, keep it at a temperature of –80°C. Just before use, add 1 g of DTT/50 mL SDS equilibration buffer. Add approx 3–4 mL of DTT-containing equilibration solutions to each strip on a strip holder tray. Place the tray on a rocker.

Before starting the procedure, make certain that all needed items are in place. Have ready the equilibration buffer (two vials/gel, 10 mL/vial) and the MW marker, which has been prepared by adding 4 µL of the Rainbow standard marker RPN 800 (MW 10,000–250,000) to the IEF sample APPL piece (Amersham Bioscience; 4 µL for silver staining and 8 µL for Commassie staining) on an ethanol-washed glass plate and allowed to dry. Cast the gel at least 3 h in advance and let it solidify. Prepare the running buffer as described in the appendix, then add running buffer to the tank and turn on the cooling unit, which usually should be between 10 and 15°C. Equilibration buffer is then prepared. Specific steps for preparation of equilibration buffer are as follows:

1. Label one equilibration buffer for DTT and the other for iodoacetamide.
2. Add 1 g DTT/50 mL buffer and 1.25 g-iodoacetamide/50 mL buffer; let them dissolve on a rocking shaker.

The process for running the second-dimension gel (SDS-PAGE) is outlined in the following steps:

1. Remove the IPG strips from the freezer, let them thaw for 3–4 min, add equilibration buffer with DTT (3 mL/strip) and lay them on a shaker for 10 min.
2. Pour out the DTT and pour in equilibration buffer with iodoacetamide. Afterwards, lay them on a shaker for 10 min.
3. Remove the strips from the equilibration buffer and wash them very carefully in running buffer.
4. Wipe off the excess running buffer on filter paper carefully, not allowing the paper to touch the gel.
5. Place the strip flush against the left end of the cassette, just touching the spacer. Place a paper with a MW marker on the end of the strip, and quickly add 5 mL preheated 1% agarose to the empty space on the gel plate and push the strip down. Wait until it is polymerized.
6. Once the agarose has polymerized, load the cassette into the tank with the hinge side on the bottom and the strip on the right.
7. Run the strip for 200 V and 7 h (running time may differ for various acrylamide concentrations and different samples).

8. When the samples have finished running, remove the gels from the cassette and place them on a gel tray filled with fixation solution.

### 3.5. Staining and Imaging of Gels

It is common to look for proteins in the gel by staining them with dye. Many different types of staining with different characteristics and limitations in respect of sensitivity for proteins are available. All stains interact differently with different proteins; thus, no stain is universal for all proteins. In our laboratory, silver nitrate staining and Coomassie blue staining are used.

Silver staining is the most sensitive nonradioactive staining method. It involves a very complex, multistep process; therefore, several variables can influence the results. To have a reproducible results, precise timing and high purity reagents are necessary. Each step in the silver nitrate staining procedure is outlined in Table 2. Destaining and restraining can reveal new spots in the lower MW area. The procedure for destining silver stained gels is as follows:

1. Mix 7.5 M potassium ferricyanide and 25 mM sodium thiosulfate together in equal parts immediately before use. Pour the mixture into the gel and put it on a shaker until all the stain is gone (approx 5 min).
2. Pour off the solution and wash with water 7 to 10 times, 3 min for each wash, until the water is no longer yellow.

<table>
<thead>
<tr>
<th>Step</th>
<th>Solution</th>
<th>Time (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Fix</td>
<td>50% methanol + 5% acetic acid</td>
<td>20</td>
</tr>
<tr>
<td>2 Wash</td>
<td>50% methanol</td>
<td>10</td>
</tr>
<tr>
<td>3 Wash</td>
<td>18 mL water</td>
<td>10</td>
</tr>
<tr>
<td>4 Sensitize</td>
<td>0.02% sodium thiosulfate</td>
<td>1</td>
</tr>
<tr>
<td>5 Wash</td>
<td>18 mL water</td>
<td>1</td>
</tr>
<tr>
<td>6 Wash</td>
<td>18 mL water</td>
<td>1</td>
</tr>
<tr>
<td>7 Silver reaction</td>
<td>0.1% silver nitrate</td>
<td>20</td>
</tr>
<tr>
<td>8 Wash</td>
<td>18 mL water</td>
<td>1</td>
</tr>
<tr>
<td>9 Wash</td>
<td>18 mL water</td>
<td>1</td>
</tr>
<tr>
<td>10 Developing</td>
<td>2% sodium carbonate + 0.04% formalin</td>
<td>Until desired intensity</td>
</tr>
<tr>
<td>11 Stop</td>
<td>5% acetic acid</td>
<td>15</td>
</tr>
<tr>
<td>12 Wash</td>
<td>18 mL water</td>
<td>5</td>
</tr>
<tr>
<td>13 Reserving solution</td>
<td>8% glycerol</td>
<td>20</td>
</tr>
</tbody>
</table>
Coomassie blue is less sensitive than silver stain (by a factor of 50), but simpler and more quantitative than the silver stain. A larger quantity of protein is needed (40 ng) in order to be detected by the Coomassie blue stain. Coomassie Blue R-250 (R for red hue) and G-250 (G for green hue) are wool dyes that are used for staining proteins in gel. Prepared Coomassie blue can be purchased, or it can be made in the laboratory. The latter must be filtered before using. Steps for staining with Coomassie blue are outlined in Table 3. Stained gels may be scanned and imaged using a scanner (we use an Epson Expression 800 scanner) with 300-dpi resolution and Photoshop (version 6.2.1, Adobe Systems Incorporated, San Jose, CA) software. The gel images are saved as .tiff files. Figure 1 shows representative Coomassie-blue stained gels from an experiment where the proteomic profile of a resistant C. albicans isolate was compared with that of its susceptible parent.

### 3.6. Data Analysis

Gel images are analyzed with PDQuest (version 7.0) 2D-gel analysis software (Bio-Rad Laboratories). Spots are detected by following the PDQuest software instructions. The parameters were adjusted until most (generally 95%) of the spots of interest were identified in the gel. The data-object (spot) is composed of individual screen pixels. The total intensity of a spot is the sum of the intensities of all of the pixels that comprise that spot. Data corresponding to experiments from which Fig. 1 was derived are shown in Table 4.

### 3.7. Protein Identification by Mass Spectrometry

For peptide mass fingerprinting using MALDI-TOF MS, we freshly prepared reagents and modifications of previously published methods (14,15).

#### 3.7.1. Spot Excision

1. Plugs from spots of interest are excised using razor blades or tips for a 1-mL pipet tip trimmed to the appropriate size.
2. Excise plugs from a nonprotein region of the gel (for use as a MS control).

---

**Table 3**

<table>
<thead>
<tr>
<th>Step</th>
<th>Solution</th>
<th>Time (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Fix 50% methanol + 5% acetic acid</td>
<td>20</td>
</tr>
<tr>
<td>2</td>
<td>Stain 0.5% Coomassie blue R-250</td>
<td>45</td>
</tr>
<tr>
<td>3</td>
<td>Destain 50% methanol + 10% acetic acid</td>
<td>Until desired intensity</td>
</tr>
<tr>
<td>4</td>
<td>Wash 18 mL water</td>
<td>10</td>
</tr>
</tbody>
</table>
3. Estimate gel volume of each sample, $1 \text{ mm}^3 = 1 \mu\text{L}$ (length $\times$ width $\times$ height for cubes, or $\pi r^2 \times$ height for cylinders; see Note 4).

### 3.7.2. Coomassie Destaining for MALDI Analysis

1. Submerge the gel spot in a 50:50 mixture of a solution containing acetonitrile:water (incubate overnight if necessary).
2. Vortex until color is removed.
3. Remove the solution.
4. Rinse the gel piece three times with 200 $\mu\text{L}$ of water.

### 3.7.3. Reduction/Alkylation

1. Add 25 $\mu\text{L}$ of 10 mM DTT/25 mM NH$_4$HCO$_3$ solution, incubate for 15 min at 65°C.
2. Return tubes to room temperature (5 min), add iodoacetamide from a concentrated stock (typically 100 mM) until the solution is 10 mM iodoacetamide.
3. Incubate for 30 min in the dark.
4. Remove the solution.
5. Wash the gel particles with 250 $\mu\text{L}$ of 25 mM NH$_4$HCO$_3$ and incubate for 15 min at room temperature.
6. Vortex gently, and then remove the solution.
7. Wash the gel particles with 250 $\mu\text{L}$ of water and incubate for 5 min at room temperature.
8. Vortex gently. Remove the solution.
<table>
<thead>
<tr>
<th>Spot number</th>
<th>Protein</th>
<th>Accession number</th>
<th>pI</th>
<th>MW (kDa)</th>
<th>Probability score</th>
<th>Estimated Z score</th>
<th>Protein coverage (%)</th>
<th>Fold change (12–99/2–79)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td><strong>Ilv5p</strong> (ketol-acid reducto-isomerase)</td>
<td>CA1983</td>
<td>6.2</td>
<td>44.83</td>
<td>1</td>
<td>2.37</td>
<td>59</td>
<td>0.6</td>
</tr>
<tr>
<td>2</td>
<td><strong>Grp2p</strong> (reductase)</td>
<td>CA2644</td>
<td>6</td>
<td>37.62</td>
<td>1</td>
<td>2.15</td>
<td>57</td>
<td>2.3</td>
</tr>
<tr>
<td>3</td>
<td><strong>Grp2p</strong> (reductase)</td>
<td>CA2644</td>
<td>6</td>
<td>37.62</td>
<td>1</td>
<td>2.32</td>
<td>43</td>
<td>2.1</td>
</tr>
<tr>
<td>4</td>
<td><strong>Ifd1p</strong> (aryl-alcohol dehydrogenase)</td>
<td>CA0840</td>
<td>5.6</td>
<td>39.14</td>
<td>1</td>
<td>2.39</td>
<td>24</td>
<td>11.2</td>
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<tr>
<td>5</td>
<td><strong>Ifd4p</strong> (aryl-alcohol dehydrogenase)</td>
<td>CA2416</td>
<td>6</td>
<td>38.26</td>
<td>1</td>
<td>2.38</td>
<td>41</td>
<td>4</td>
</tr>
<tr>
<td>6</td>
<td><strong>Ifd5p</strong> (aryl-alcohol dehydrogenase)</td>
<td>CA0924</td>
<td>5.4</td>
<td>39.20</td>
<td>1</td>
<td>2.27</td>
<td>39</td>
<td>9.3</td>
</tr>
<tr>
<td>7</td>
<td><strong>Ifd6p</strong> (aryl-alcohol dehydrogenase)</td>
<td>Ca2417</td>
<td>5.9</td>
<td>39.06</td>
<td>1</td>
<td>2.35</td>
<td>29</td>
<td>&gt;10</td>
</tr>
</tbody>
</table>

pI, isoelectric point; MW, molecular weight.
3.7.4. **Mincing and Dehydration of Gel Particles for Silver Stain**

1. Cube the gel plugs into 1-mm³ cubes using a clean blade, then add 2 gel volumes of water. Add 2 gel volumes of acetonitrile (vortex), incubate for 5 min at 0°C.
2. Dry down the gel particles in a vacuum centrifuge.

3.7.5. **Rehydration and In-Gel Digestion**

1. Add 3 gel volumes of a digestion buffer containing: 25 mM NH₄HCO₃, 5% acetonitrile (ACN), 38 ng/µL trypsin. Vortex the samples, quick spin (at 4°C) to submerge all particles, then incubate for 15 min at 0°C.
2. Repeat the vortex and spin steps from step 1, and incubate at 0°C for 3–6 h.
3. Remove excess supernatant.
4. Top off the gel pieces with 2 gel volumes of a 25 mM NH₄HCO₃, 5% ACN solution.
5. Incubate the samples overnight at 37°C.

3.7.6. **Extraction of Peptides**

1. Sonicate for 2 min, vortex for 5 min, and centrifuge for 2–3 min (20,000g at 4°C) then transfer the supernatant to a new microfuge tube. Keep the supernatant on ice.
2. Extract three more times with an acetonitrile:water (50:50) solution containing 5% trifluoroacetic acid (TFA). In each extraction, sonicate for 2 min, vortex for 5 min and spin for 2–3 min (20,000g at 4°C).
3. Combine all supernatants with the first.
4. Clarify the total supernatant with a hard spin for 10 min (20,000g at 4°C) and transfer supernatant to a new microfuge being careful to leave any pellet (acrylamide bits) in old tube.
5. Spin the samples to dryness to remove NH₄HCO₃/TFA using a vacuum centrifuge.
6. Add water, vortex, and spin to dryness using a vacuum centrifuge to remove NH₄HCO₃/TFA (overnight).

3.7.7. **Desalting of Samples With Zip Tips®**

1. Prepare the following solutions:
   a. Wetting solution: water:acetonitrile (1:1).
   b. Equilibration and wash solution: 0.1% TFA, 3% acetonitrile in water.
   c. Elution solution: 70% acetonitrile, 0.1% TFA in water.
2. Reconstitute sample in 20 µL of equilibration solution.
3. Slowly aspirate 20 µL of wetting solution into a Zip Tip and dispense to waste.
4. Repeat several times.
5. Slowly aspirate 20 µL of equilibration solution (0.1% TFA in water) into the Zip Tip and dispense to waste.
6. Repeat several times.
7. Allow sample to bind to Zip Tip by repeatedly drawing and dispensing 20 µL of sample solution (slowly, about 10–15 times) into the sample tube, expel the last of the solution back into the sample tube leaving the Zip Tip empty.
8. Wash Zip Tip by slowly drawing and dispensing 20 µL of wash solution 1X to waste.
9. Elute sample from Zip Tip by slowly drawing and dispensing approx 5 µL of elution solution into a clean microfuge tube.
10. The 5-µL sample is ready for MALDI-MS analysis.

3.7.8. Sample Preparation for MALDI Analysis
1. Prepare matrix using 990 mL of acetone and 10 µL in 0.1% TFA in water, then add 0.03 g of α-cyano-4-hydroxycinnamic acid.
2. Clean the MALDI target plate by rubbing with a Kim wipe and 50 µL MeOH, acetonitrile, and water, respectively.
3. Transfer a 0.5-µL droplet of sample to a well on the MALDI target plate.
4. Immediately add 0.5-µL droplet of matrix to the sample droplet on the target plate.
5. Record mass spectra of sample immediately after droplet is dry.

3.8. Spot Identification Through Custom Database Searching
Proteins can be identified by searching in appropriate databases with peptide mass fragmentation data. Mass fingerprints for unknown proteins are generated using MALDI-TOF MS. PROWL software (formerly Proteometrics, Inc.) is used to search protein databases with mass spectrometry data. A custom database for PROWL has been constructed in our laboratory from a database of Candida albicans open reading frames (ORFs) DNA sequence (http://genolist.pasteur.fr/CandidaDB/), and is used to match unknown C. albicans proteins to the specific ORFs in the organism. A probability score for the match is attained in PROWL, with an accompanying Z score that represents a goodness of fit of the probability score for the search result. A Z score of 1.65 ranks the search result in the 95th percentile of nonrandom matches of the mass dataset to the specific ORF.

4. Notes
1. It is very important to remove as many salts as possible, because these salts will affect the first dimension isoelectric focusing, and, ultimately, will affect 2D-gel results.
2. C. albicans cells do not disrupt easily; therefore, a more vigorous lysis method should be used in order to complete disruption of their cells. It is important to avoid heating the cells or producing foam.
3. It is usually preferable to load the sample with the rehydration buffer solution, because this allows large quantities of protein to be loaded and separated. It also allows time for high-MW proteins to enter to the gel as it becomes fully hydrated. This procedure is also simpler to perform. The IPG strip should return to its original thickness (0.5 mm) before use.
4. Prepare “MS clean” microfuge tubes for all steps to eliminate background ions in mass spectra by washing microfuge tubes three times with a solution containing: acetonitrile:water:glacial acetic acid (45:45:10, respectively) prior to use.
References


Techniques for Antifungal Susceptibility Testing of Candida albicans Biofilms

Gordon Ramage and José Luis López-Ribot

Summary

Candida albicans is capable of forming biofilms on a variety of inert and biological surfaces. Cells in biofilms display phenotypic properties that are radically different from their free-floating planktonic counterparts, including their recalcitrance to antimicrobial agents. Consequently, Candida biofilm-associated infections are difficult to treat and to fully eradicate with standard antifungal therapy. Here, we describe a simple, fast, inexpensive and highly reproducible microtiter plate-based assay for the antifungal susceptibility testing of C. albicans biofilms. Because of its simplicity, compatibility with widely available 96-well microplate platform, high throughput, and automation potential, this assay represents an important tool towards the standardization of in vitro antifungal susceptibility testing of fungal biofilms.

Key Words: Candida albicans; fungal infections; biofilms; resistance; sessile cells; antifungal drugs; susceptibility testing; standardization; microtiter plate; formazan salt; colorimetric assay.

1. Introduction

Biofilms are defined as structured microbial communities that are attached to a surface and encased in a matrix of exopolymeric material (1). A wide range of biomaterials used in clinical practice support colonization and subsequent biofilm formation by Candida spp. (2–4). Indeed, the increase in Candida infections in the last few decades has paralleled the widespread use of many medical implant devices (5). One important consequence of biofilm formation is the increased resistance to antifungal therapy, with serious clinical repercussions (6–8).

It was not until recently that the National Committee for Clinical Laboratory Standards (NCCLS) published its guidelines for a standardized broth macro- and microdilution assay for in vitro testing of antifungal susceptibili-
ties (9). Results that are obtained using this methodology exhibit good in vitro–in vivo correlation, particularly in the setting of oropharyngeal candidiasis in infected individuals with human immunodeficiency virus (10). However, this method for susceptibility testing does not take into account that biofilm (sessile) cells are phenotypically different and are numerically greater than free-floating (planktonic) cells used for standardized NCCLS testing. Consequently, for suspected biofilm-related infections, NCCLS standardized testing does not provide an accurate in vitro–in vivo correlation.

This chapter describes in detail the techniques required for a rapid, inexpensive, easy-to-use, accurate, and reproducible methodology for antifungal susceptibility testing of Candida albicans biofilms (11), which also can be adapted to demonstrate the pharmacokinetic killing profiles of antifungal agents against preformed biofilms (12). This method is based on the formation of multiple, equivalent C. albicans biofilms on the bottom of wells of microtitre plates and on the use of a colorimetric method to measure metabolic activity of sessile cells within the biofilms as it relates to cellular viability. The assay is based on the reduction of 2,3-bis(2-methoxy-4-nitro-5-sulfo-phenyl)-2H-tetrazolium-5-carboxanilide (XTT), a tetrazolium salt that, on reduction by dehydrogenase enzymes, yields a water-soluble colored formazan product that can be measured using a microtiter plate reader (13,14). This colorimetric assay is used in preference to viable cell counting because of its nondestructive nature and minimal postprocessing of samples after antifungal challenge.

2. Materials

1. Clinical or laboratory strains of C. albicans.
2. Yeast peptone dextrose (YPD) agar plates for subculturing Candida isolates: 1% w/v yeast extract, 2% w/v peptone, 2% w/v dextrose, 1.5% agar (US Biological, Swampscott, MA).
3. YPD liquid medium: 1% w/v yeast extract, 2% w/v peptone, 2% w/v dextrose.
4. RPMI-1640 supplemented with L-glutamine and buffered with morpholinepropanesulfonic acid.
5. Sterile phosphate-buffered saline (PBS): 10 mM phosphate buffer, 2.7 mM potassium chloride, 137 mM sodium chloride, pH 7.4.
6. Hemocytometer.
7. Polystyrene, flat-bottomed, 96-well microtiter plates
8. XTT.
10. Ringer’s lactate.
12. Bright field inverted microscope.

3. Methods

The methods described under the following subheadings outline:

1. The formation of *C. albicans* biofilms.
2. The procedures for antifungal susceptibility testing of cells within biofilms.
3. The demonstration of antifungal kinetic profiles.

3.1. Biofilm Formation

This section describes the formation of *C. albicans* biofilms in microtiter plates, including the following:

1. The initial growth and storage of the micro-organism(s).
2. Standardizing the initial inoculum of yeast cells for biofilm formation
3. Seeding the plates and growth of *Candida* biofilms.

3.1.1. Storage and Growth of Candida Isolates

*Candida* isolates are typically stored either as glycerol stocks or on Sabouraud dextrose slopes (BBL, Cockeysville, MD) at –70°C. Some laboratories keep their yeast collections as suspensions in water at room temperature. For propagation of *Candida* organisms, a loopful of cells from the stocks (or a fresh culture from a clinical sample) is streaked onto YPD agar, which is incubated overnight at 37°C. Flasks containing YPD liquid medium (typically 20 mL of medium in a 150-mL flask) are then inoculated with a loopful of cells from the YPD agar plates containing freshly grown isolates, and incubated overnight in an orbital shaker (150–180 rpm) at 30°C. Typically, all *C. albicans* isolates grow in the budding–yeast phase under these conditions.

3.1.2. Standardizing the Candida Inoculum

This step involves washing and standardizing the yeast inoculum for seeding the wells of microtiter plates for the initial biofilm formation (see Note 1).

1. Cells are harvested from the overnight grown liquid cultures and washed twice by centrifugation (3000g) in sterile PBS.
2. Cells are then resuspended in RPMI-1640 supplemented with L-glutamine and buffered with morpholinepropanesulfonic acid (Angus Buffers and Chemicals, Niagara Falls, NY).
One hundred- and one thousand-fold dilutions of the resulting cell suspension are prepared and counted using hemocytometer on a bright-field microscope with a 40× objective lens (see Note 2).

After counting, a final yeast suspension is prepared at a cell density of $1 \times 10^6$ cells/mL in RPMI and processed immediately (see Note 3).

### 3.1.3. Biofilm Formation

1. *C. albicans* biofilms are formed on commercially available presterilized, polystyrene, flat-bottomed, 96-well microtiter plates (Corning Incorporated, Corning, NY).
2. Biofilms are formed by pipetting standardized cell suspensions (100 µL of the $1 \times 10^6$ cells/mL) into selected wells of a microtiter plate.
3. The 12th column of wells on the plate should remain empty because these eight wells will act as negative background controls during subsequent analysis and quantification.
4. The microtiter plate is covered with its lid, sealed with parafilm, and incubated for 24–48 h at 37°C (see Note 4).

### 3.1.4. Postbiofilm Formation Processing

1. After biofilm formation the RPMI medium is aspirated (see Note 5).
2. Planktonic and nonadherent cells are removed by thoroughly washing the biofilms three times in sterile PBS (200 µL per well).
3. Residual PBS is removed by blotting with paper towels before the addition of antifungal agents. Biofilms are now ready to be processed for antifungal susceptibility testing assays.

### 3.1.5. Visual Analysis of Preformed Biofilms

1. After the incubation period, biofilms formed on the bottom of the wells are visible to the naked eye, simply by looking at the underside of the microtiter plate.
2. A bright-field microscope or an inverted microscope with a 20× or 40× objective is sufficient to examine morphological details of the formed biofilms.
3. Images can be captured if microscope contains attachments that would allow for image acquisition (conventional or digital photography and video equipment).

### 3.2. Determination of the Effect of Antifungal Agents on Preformed *C. albicans* Biofilms

Here, we will discuss the following:

1. Preparation of antifungal agents for testing.
2. Challenging intact *C. albicans* biofilms with antifungal agents.
3. Use of a colorimetric metabolic assay as a semiquantitative method to assess fungal cell viability after treatment with antifungal agents.
4. Interpretation of the sessile minimum fungicidal concentrations.
3.2.1. Preparation of Antifungal Agents for Biofilm Testing

1. Antifungal agents are selected based on the testing required.
2. The drugs are solubilized following manufacturer’s instructions. High concentrations of antifungals are needed.
3. If needed, the antifungals can be aliquoted into smaller volumes and stored at −70°C until required.
4. Final working concentrations of each antifungal (in the range of 20–1000× the predicted planktonic minimum inhibitory concentrations) are prepared in RPMI medium. Typical high concentrations for fluconazole are 1024 µg/mL and 16 µg/mL for both amphotericin B and caspofungin.

3.2.2. Challenging Preformed Candida Biofilms With Antifungal Agents

1. 200 µL of the high working concentration of antifungal is added to each of the eight wells on the first column of the microtiter plate containing fungal biofilms, being careful not to disrupt the biofilms (see Note 6).
2. 100 µL of RPMI per well is added to columns 2–10.
3. 200 µL of RPMI is placed in column 11 as the positive control (biofilm not exposed to antifungal), and column 12 remains empty as the negative control.
4. 100 µL of antifungal agent in the wells of column 1 are then removed and added to the RPMI of adjacent column 2 wells.
5. The contents are mixed by pipeting gently up and down, to perform a serial doubling dilution, and the pipet tips removed.
6. Step 5 is repeated up to the wells of column 10, after which the final 100-µL volume is discarded.
7. The plates are then covered with their lids, sealed with parafilm, and incubated for 24–48 h at 37°C.
8. After antifungal challenge, biofilms are processed and washed as described in Subheading 3.1.3. The plates are now ready for the addition of XTT reagent.

3.2.3. Use of a Colorimetric Metabolic Assay (XTT Reduction Assay) as a Semiquantitative Method to Assess Fungal Cell Viability After Treatment With Antifungal Agents

The effect of antifungal treatment on *C. albicans* biofilms is estimated using a soluble metabolic dye (XTT). This approach is advantageous over traditional approaches to quantifying microbial biofilms, which require postprocessing of the biofilm, for example, disaggregation, sonication, and total viable cell counting, because these techniques are susceptible to miscalculation of the biofilms due to clumping and poor technique. This section outlines the solutions required for the assay and the estimation of metabolic activity of cells within biofilms.
3.2.3.1. Preparation of XTT Reduction Assay Solution (see Note 7)

1. XTT (Sigma) is prepared in a saturated solution at 0.5 g/L in Ringer’s lactate (see Notes 8 and 9).
2. The XTT/Ringer’s lactate solution is filter sterilized through a 0.22 m-pore size filter, aliquoted into 10-mL working volumes, and stored at −70°C. Tubes of the XTT solution are wrapped in aluminium foil to prevent light penetration (see Notes 8 and 9).
3. Menadione (Sigma) is prepared as a 10 mM stock solution in 100% acetone, and stored at −70°C.
4. Before each assay, an aliquot of stock XTT is thawed and menadione added to a final concentration of 1 µM (1 µL per 10 mL of XTT solution).

3.2.3.2. Quantification of the Metabolic Activity of Cells Within Biofilms

1. A 100-µL aliquot of the XTT/ menadione solution is added to each pre-washed biofilm and to negative control wells (for the measurement of background XTT-colorimetric levels).
2. The plates are then covered in aluminium foil and incubated in the dark for up to 2 h at 37°C (see Note 10). If XTT is effectively reduced by live yeast cells, the originally clear solution is transformed into an orange color.
3. After incubation of the microtiter plates, 75 µL of XTT dye from each well is removed and transferred to the wells of a brand new microtiter plate (without biofilms).
4. These plates are then read in a microtiter plate reader (Benchmark Microplate Reader, Bio-Rad, Hercules, CA) at 490 nm.

3.2.4. Interpretation of the Minimum Fungicidal Concentrations

1. The sessile minimum inhibitory concentrations (SMICs) are defined as the antifungal concentrations at which a 50% (SMIC50) or 80% (SMIC80) reduction in XTT-colorimetric readings in comparison to the unchallenged control biofilms are measured (see Note 11).
2. Alternatively, visual inspection of the plate can sometimes be employed to determine the efficacy of the antifungal drug tested (see Note 12).

3.3. Adaptation of This Technique to the Study of Time-Killing Kinetics of Antifungal Agents Against C. albicans Biofilms

In addition to the determination of SMICs, it is possible to monitor the kinetics of the effects of antifungal drugs against intact biofilms over a period of time (11). The methodology described in Subheadings 3.1.–3.3., can be easily adapted to the analysis of the rate at which various antifungal drugs kill intact biofilms. Here we will describe the simple steps involved in this assay.
3.3.1. Biofilm Preparation

1. Biofilms are prepared in microtiter plates in an identical manner to those described in Subheading 3.1.
2. Multiple wells in microtiter plates with replicate biofilms are prepared for each time point to be tested.
3. Biofilms are grown for 24–48 h at 37°C.
4. Biofilms are then washed gently in sterile PBS before the addition of antifungal agents.

3.3.2. Antifungal Agents

1. Antifungal agents and their concentrations are decided before commencing the experiment.
2. Typically, selected concentrations of antifungal agents are determined at sensitive, intermediate, and resistant ranges, based on NCCLS minimum inhibitory concentrations (see Note 13).
3. These concentrations are prepared in RPMI, as described in Subheading 3.2.1.

3.3.3. Antifungal Challenge

1. 200 µL of antifungal agent are added to preformed biofilms in each microtiter plate using eight replicate biofilms for each concentration tested.
2. Biofilms are challenged for selected time intervals (1, 2, 4, 6, 8, 24, and 48 h) and incubated at 37°C.
3. After antifungal challenge for the selected time interval, antifungals are decanted gently and biofilms washed in PBS.
4. XTT is then added to the biofilms, as described in Subheading 3.2.3., and the metabolic activity of the biofilms assessed after a 1-h incubation by means of the colorimetric method.
5. Curves of killing kinetics are typically generated by plotting the percentage reductions in XTT-colorimetric readings (in challenged biofilms compared with untreated control biofilms) vs time (hours). A logarithmic scale is recommended.

4. Notes

1. The yeast cell density is very important in relation to biofilm formation. Densities that are too high or too low result in poorly formed biofilms. The inoculum $1 \times 10^6$ cells/mL has been empirically optimized for these experiments in 96-well microtiter plates.
2. If a hemocytometer and microscope are unavailable, it is possible to determine the cellular density by performing total viable cell counts at varying absorbance readings to create a standard curve, which can be used for future experiments, if performed in an identical manner. Alternatively, a McFarland standard may be used to estimate cell density and to estimate cell numbers.
3. It is important that the yeast cells are not left sitting on the bench for an extended period of time because they (particularly in the case of *C. albicans*) could begin to form hyphae in the RPMI, which may affect their initial adhesion to the microtiter plate. It is encouraged that inoculation of the wells occurs promptly after counting and processing.

4. The duration of biofilm formation may be varied depending on the specific study. In our own observations, 24-h biofilms already display the typical architecture and three-dimensional structure that is characteristic of “mature” biofilms and can be used for antifungal susceptibility testing. Also, the incubation period for biofilm formation can be changed according to the purpose of the study: for example, if examination of initial adherence is desired the incubation period is much reduced.

5. Aspiration of the media from the biofilm must be performed carefully as not to touch and destroy the biofilm. Using a multichannel pipet, angle the pipet tips toward the corners of the wells because this minimizes contact with the biofilm. Alternatively, the biofilms are adhered well to the bottom of the wells, so by flicking the wrist, it is possible to remove the media in a constant action (this requires some practice and skill). When multiple strains are being used, the latter technique is discouraged as contamination may result.

6. We strongly advise the use of a multichannel pipet for these manipulations.

7. The metabolic reduction assay is reliant on the mitochondrial dehydrogenases of the live cells to covert a XTT tetrazolium salt into a reduced soluble formazan colored product that can be measured spectrophotometrically.

8. Ringer’s lactate can be exchanged for PBS or any other physiological buffer and has been shown to give similar reproducible results.

9. XTT solution is light sensitive, so it should be covered with aluminium foil during preparation. As it is a saturated solution, the filtration step will leave yellow residues on the filter, this does not constitute a problem. Also, storage of XTT for prolonged periods (longer than 1 yr) is not advised because the activity of the reagent may decrease over time.

10. The incubation time is variable. We optimized for 2 h for our *C. albicans* strains; however, some other strains and *Candida* species could be less metabolically active.

11. We advise that if a large experiment is prepared, XTT should be prepared fresh, menadione added, and stored in the fridge (no longer than 3 d). This preparation allows comparative studies from the same batch of XTT. Batch-to-batch variation does occur, but if proper intra-experimental controls are used, then it will not problematic in the interpretation of the results. We do not advise direct comparisons of absorbance readings from separate experiments.

12. Visual inspection of the plates will demonstrate an orange color gradient, which depending on the antifungal used, will show a distinct efficacious cut-off concentration.

13. Antifungal concentrations that are based on individuals own preference.
References
Natural Products and Antifungal Drug Discovery

Melissa R. Jacob and Larry A. Walker

Summary

The need for new antifungal agents continues, fueled by opportunistic infections in immunocompromised patients and by the development of resistance to existing agents. Natural products offer a virtually unlimited source of unique molecules and not only serve as a reservoir for new potential drugs and drug prototypes, but also for probes of fungal biology. In this chapter, whole-cell screening methods targeted for natural products are illustrated, including general microplate-based screening, bioautography, and mode of action studies, including the use of genetically altered fungal strains now available commercially.

Key Words: Antifungal; susceptibility testing; natural products; whole-cell bioassay; *Candida albicans; Cryptococcus neoformans; Aspergillus; Saccharomyces cerevisiae*; target-based screening; bioautography; tannins; haploinsufficiency; synergy.

1. Introduction

The healing properties of plants and animals have been recorded for thousands of years in such documents as Dioscorides’ “de Materia Medica” from 75 AD and the 3500-yr-old *Papyrus Ebers* (1). Fast forward four millennia and natural products, although ensconced in more sophisticated drug-delivery systems, continue to help humankind. Modern examples include lovastatin, digi- toxin, reserpine, morphine, and cyclosporin A (2). Moreover, plants (3–5), marine organisms (6,7), and microbes (8,9) usually produce biologically active compounds in defense to predators and competition with neighbors (10). Thus, it seems logical that most of the drugs derived from natural sources have anticancer or anti-infective properties (11). Just a few examples include vincristine, taxol and camptothecin (anticancer), quinine and artemisinin (antimalarial), tetracyclines, macrolides, and cephalosporins (antibacterial), and polyenes, echinocandins/pneumocandins, aureobasidins and sordarins (antifungals) (8,12).
Although Sir Fleming’s discovery of penicillin (13) (and more ancient findings) was somewhat serendipitous, and a little good fortune may still play a role in finding new drugs, antimicrobial drug discovery is more systematic and elaborate in the 21st century, but so are pathogenic microbes. Fungal and bacterial infections persist as a threat to worldwide health and cost the world billions of dollars each year. Drug companies continue to focus on the development of antimicrobial drugs, especially with the increasing emergence of drug-resistant pathogens (14). Natural products are just one source of antimicrobial agents among today’s world of chemical libraries and combinatorial syntheses, but they offer an almost unlimited reservoir of unique structures. In fact, many of the aforementioned compounds were discovered before their mechanism of action, paving the way for the use of natural molecules as probes for undiscovered biological processes (15). Coupled with complete genome sequences of target and surrogate organisms, antimicrobial drug discovery from natural products is entering a new era. This chapter is designed to provide an overview of several methods adapted for whole-cell screening of natural products for the discovery of antifungal compounds.

2. Materials

All manipulations with micro-organisms should be conducted using standard aseptic techniques under an appropriate biological safety cabinet. All media are prepared as directed and sterilized either by filtration or autoclaving.

1. Appropriate fungi include the American Type Culture Collection reference strains (Candida albicans ATCC 90028,Cryptococcus neoformans ATCC 90113, Aspergillus fumigatus ATCC 90906), clinical isolates, Saccharomyces cerevisiae-deletion/resistant mutants, and appropriate wild types, many of which are available from the ATCC (Manassas, VA), Research Genetics/Invitrogen Life Technologies (Carlsbad, CA), and Open Biosystems (Huntsville, AL) provided:
   a. Frozen in 10–20% glycerol/broth stocks.
   b. As dehydrated cultures.
   c. On agar plates or slants.
2. Various broths and agars, including rich media (Sabouraud dextrose [SD], yeast peptone dextrose [YPD]), synthetic/defined media (RPMI-1640, yeast nitrogen base [YNB]).
3. Media supplements, including glycerol, dextrose, amino acids, nitrogenous bases (available premixed from companies such as Mediatech, Herndon, VA), Alamar Blue™ (BioSource International, Camarillo, CA), and buffering agents, including 3-(N-morpholino) propanesulfonic acid (MOPS).
4. Filter sterilization units.
5. 96-Well, flat-bottom, clear microplates/plate sealers and Petri dishes (various sizes).
6. Drug controls, for example, amphotericin B (ICN Biomedicals, Costa Mesa, CA), fluconazole (Pfizer, Morris Plains, NJ), or other appropriate positive controls.
7. 0.9% Sodium chloride (NaCl).
8. Solvents: dimethylsulfoxide (DMSO), ethanol (EtOH), polyethylene glycol (PEG), or others innocuous to test organisms and conditions.
9. Sterile tips and pipetors (electronic, manual, 8-, 12-, single-channel) capable of accurately delivering 1–1000 µL.
10. Inoculating loops, cell spreaders, and sterile cotton applicators.
11. 96-Well microplate readers capable of monitoring absorbance and fluorescence.
12. Hemacytometer and cover slips.
13. 0.5 McFarland Standard: 1:200 dilution of 48 mM BaCl₂ with 180 mM H₂SO₄.
14. Filtration material suitable for 20- to 25-µ particle size (e.g., Miracloth®, Calbiochem, La Jolla, CA).
15. Thin-layer chromatograms (TLCs), TLC chambers, chromatography sprayer/atomizer, solvent systems, ultraviolet source, and spray reagents (i.e., vanillin/H₂SO₄).
17. Tannic acid, gelatin, and ferric chloride (FeCl₃).

3. Methods

The bulk of the literature regarding natural product screening in the late 20th century primarily describes agar well and diffusion methods. These techniques do detect active extracts, but in a medium- to high-throughput antifungal screening laboratory these methods are relatively cumbersome. Along with broth macrodilution techniques, agar methods require relatively large amounts of sample, which may limit the use of natural products (17,18). These methods are therefore not described here but can be referred to in the following references (19–21). Microplate-based screening is more desirable when evaluating hundreds to thousands of samples/week, requires as little as nanogram–microgram quantities of test sample, and is amenable to automation.

Currently, successful antifungal drugs used clinically for systemic infections affect four general biological processes: ergosterol synthesis or function (allylamines, azoles, and polyenes), deoxyribonucleic acid (DNA) and protein synthesis (5-flucytosine), and glucan synthesis (caspofungin). The ideal antifungal drug target is one that inhibits an essential process in a broad spectrum of pathogenic fungi and has no analogous counterpart in humans, and estimates from studying the \textit{C. albicans} and \textit{S. cerevisiae} genomes reveal that 100–200 genes (approx 2.5%) fall into this category (22). Whether a screening program centers on an existing drug target that has already been validated or is aimed at new targets, a subcellular assay (such as monitoring enzyme inhibi-
(22) is usually needed to characterize the mechanism of potential drug candidates. Several methods are available for probing fungal drug targets (23–27); however, screening crude plant extracts in isolated enzyme assays is generally not advisable because of false positives owing to the presence of tannins and other nonspecific inhibitors (see Note 1; Table 1, Fig. 1 [28–30]). Subcellular/ enzymatic assays are usually therefore reserved for pure compounds as adjuncts to whole cell assays. It should be mentioned that it is possible, with some targets, to develop cellular assays that are more mechanistically specific. For example, yeast cells may be engineered with reporter genes linked to a specific promoter relevant for particular mechanistic pathways in fungi and mammals (31,32). Examples of cellular, yet mechanism-based assays, include sterol synthesis inhibition (33), assessment of estrogenic activity (34), and other techniques presented in this chapter (see Subheadings 3.1.2. and 3.3.). The following methods outline whole-cell methods amenable to screening natural product extracts and fractions and include general microplate-based screening, bioautography, and mode-of-action studies.

A whole-cell assay usually is the primary screening approach used to generate high-priority extracts/fractions for further study (bioassay-directed fractionation). This approach has the advantage of simplicity, cost-effectiveness, direct relevance to desired antifungal action, and compatibility with assay of crude extracts. Although there is no single accepted method for the whole-cell antifungal evaluation of natural products, a screening program should employ sound techniques that standardize the bioassay process, such as those described by the National Committee for Clinical Laboratory Standards (NCCLS [35,36]). Many aspects of the screening process may be adapted from the NCCLS methods to accommodate the variety of samples found with natural products. However, evaluation of pure compounds is more amenable to suggested NCCLS methods allowing direct comparison with literature values of clinically used antifungals. Methods should be consistent and basic experimental conditions remain within acceptable ranges so that intersample and interassay comparisons are possible.

### Table 1

<table>
<thead>
<tr>
<th>Sample</th>
<th>Glucosyltransferase % inhibition</th>
<th>IC$_{50}$ vs protease (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>85</td>
<td>15</td>
</tr>
<tr>
<td>Tannin-poor fraction</td>
<td>35</td>
<td>&gt;50</td>
</tr>
<tr>
<td>Tannin-rich fraction</td>
<td>72</td>
<td>6</td>
</tr>
</tbody>
</table>

IC, concentration that affords 50% inhibition of enzyme activity.
Fig. 1. (A) Comparison of percent inhibitions of a fungal protease by crude extracts with low to high tannin indices (see Note 1). (B) Tannin analysis using tannic acid. Tannins have the ability to both precipitate proteins (cloudiness in 10%/1% NaCl/gelatin wells) and react with FeCl$_3$ to form a blue, gray, or green color (0.2% FeCl$_3$ wells).

DMSO, dimethylsulfoxide.
<table>
<thead>
<tr>
<th>Step</th>
<th>Candida albicans</th>
<th>Cryptococcus neoformans</th>
<th>Aspergillus fumigatus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample preparation</td>
<td>Weighing antifungal powders on an analytical balance (taking into account the potency/purity) and dissolving in water to obtain a 10X solution (or 100X if solvents other than water such as DMSO, EtOH, or PEG are used)</td>
<td>Serial dilution of the sample in its solvent to afford 10X (or 100X for nonaqueous solvents) final test concentrations to incorporate expected breakpoints with subsequent 1:5 dilution in broth</td>
<td></td>
</tr>
<tr>
<td>Strain selection</td>
<td>ATCC 90028</td>
<td>ATCC 90113</td>
<td>ATCC 90906</td>
</tr>
<tr>
<td>Inoculum preparation</td>
<td>Suspension of colonies from a 24-h agar culture is prepared in 0.9% saline, corrected to the 0.5 McFarland, and diluted 1000-fold in broth to afford 2X final inoculum; a 1:1 ratio sample dilution:organism produces a final inoculum of 0.5–2.5 × 10³ CFU/mL</td>
<td>Suspension of colonies from a 48-h agar culture is prepared in 0.9% saline, corrected to the 0.5 McFarland, and diluted 1000-fold in broth to afford 2X final inoculum; a 1:1 ratio sample dilution:organism produces a final inoculum of 0.5–2.5 × 10³ CFU/mL</td>
<td>Suspension of conidia from a 7-d agar culture is prepared in 0.9% saline, corrected to 0.09–0.11 OD, and diluted 50-fold in broth to afford 2X final inoculum; a 1:1 ratio sample dilution:organism produces a final inoculum of 0.4–5.0 × 10⁴ CFU/mL</td>
</tr>
<tr>
<td>Broth selection</td>
<td>RPMI-1640 + MOPS @ pH 7.0 (2% glucose may be added)</td>
<td>RPMI-1640 + MOPS @ pH 7.0 (2% glucose may be added); YNB broth may be substituted</td>
<td>RPMI-1640 + MOPS @ pH 7.0 (2% glucose may be added)</td>
</tr>
<tr>
<td>Incubation</td>
<td>46–50 h @ 35°C</td>
<td>70–74 h @ 35°C</td>
<td>46–50 h @ 35°C</td>
</tr>
<tr>
<td>End point determination</td>
<td>Minimum inhibitory concentration based on a visual score</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

From refs. 35,36.

ATCC, American Type Culture Collection; DMSO, dimethylsulfoxide; EtOH, ethanol; PEG, polyethylene glycol; OD, optical density; CFU, colony-forming units; MOPS, 3-(N-morpholino) propanesulfonic acid.
3.1. General Microplate-Based Whole-Cell Screening

3.1.1. Sample Preparation and Sample Dilution

Table 2 illustrates the general methods recommended by the NCCLS for antifungal susceptibility testing (35,36). Each step may be adapted to encompass a natural product screening laboratory’s desired outcome. Unfortunately, when dealing with crude extracts and column fractions of natural products, purity and potency of the sample are not applicable. However, careful attention to consistency of sample preparation (collection, weighing, extraction, storage, dissolution) will minimize these sources of variability or inconsistency. The solvent used should be one that is able to dissolve a variety of sample types (water is therefore not usually successful, especially when dealing with organic extracts) and innocuous to the test organism and conditions. DMSO and EtOH are possible alternatives. However, the hygroscopicity of DMSO and volatility of EtOH may affect the ultimate sample concentration.

The screening process can be organized in a hierarchical format using an increasing number of test concentrations with increasing purity of the sample (see Notes 2 and 3; Fig. 2).

1. Accurately weigh extract, column fraction, or pure compound using an analytical balance or aliquotting a known volume of a known concentration of the sample completely dissolved in a volatile solvent (i.e., methanol, chloroform) and allowing to dry.
2. Dissolve the dried sample in DMSO to at least 100× the final desired test concentration.
3. Dilute dissolved sample in assay medium (see Note 4) preparing serial dilutions if desired (see Note 2).
4. Transfer diluted samples in duplicate to 96-well flat bottom microplates for assay.
5. Add inoculum (see Subheading 3.1.3.) to all sample containing wells. Include a media (diluent + broth) and solvent (equivalent concentration of solvent + inoculum) control on each microplate (see Note 4).

3.1.2. Strain Selection

For general antifungal screening, the NCCLS-recommended reference strains should be used because they have been shown to be genetically stable, and positive controls (e.g., amphotericin B) should be included with each assay to monitor the organism’s susceptibility. Any deviation from normal activity ranges indicates that either new drug controls or new organisms should be prepared.

In lieu of recommended reference strains, genetically altered strains may be used in a standard microplate assay. Gene dosage effects (deletion or overexpression of genes) have been shown to be useful in determining the mode of action of bioactive compounds (37–41), and an almost complete deletion set
Fig. 2. Comparison of antifungal activity to growth media of (A) crude extracts and (B) pure compound. (A) Dose–response curves of two crude extracts in three broth conditions: 1. RPMI-1640 + 2% glucose buffered with 3-(N-morpholino) propanesulfonic acid (MOPS) at pH 7.0; 2. Sabouraud dextrose (SD) broth buffered with MOPS at pH 7.0; 3. SD broth unbuffered at pH 5.75. (B) Dose–response curves of a diterpene containing a carboxylic acid functionality in SD broth unbuffered at pH 5.75 and RPMI-1640 + 2% glucose buffered with MOPS at pH 7.0.
of *S. cerevisiae* is available from Research Genetics/Invitrogen, Open Biosystems, and ATCC (42). Complete knockouts, as well as haploinsufficient strains (one gene copy), are available. Side-by-side analysis with the isogenic wild-type strain should be conducted. The rationale is that increased susceptibility of the haploinsufficient strain or enhanced resistance of the overexpression mutant strain relative to the wild type is consistent with the gene’s product being involved in the mechanism of action. However, genes associated with nonspecific actions such as drug efflux or enhanced permeability also may be implicated and should be taken into consideration. Additionally, in some instances, the overexpression of a drug target may result in increased susceptibility, as with some topoisomerase inhibitors, depending on the mechanism of inhibition (43).

### 3.1.3. Inoculum Preparation

The inoculum size has been shown to affect the activity of test samples (generally, the higher the inoculum, the more sample required to inhibit at equivalent levels [44]). Moreover, it may be necessary to decrease the incubation time when higher inocula are used. Therefore, in a screening laboratory where samples are tested regularly, the inoculum size should be within an acceptable range with each assay. The inoculum is defined as the number of viable cells (colony-forming units [CFU]) per milliliter and can be estimated using several methods.

#### 3.1.3.1. Hemacytometer Counts

This method determines a cell/mL measurement of a dilute suspension of the test organism in 0.9% saline by counting on a hemacytometer.

1. Resuspend colonies in 0.9% saline (see Note 5, Table 3, Fig. 3).
2. Dilute cell suspension 100× with 0.9% saline.
3. Apply approx 10 μL of the diluted suspension to a hemacytometer and apply a cover slip.
4. Count the number of cells at 10× or 40× magnification according to the hemacytometer instructions.
5. Calculate the number of cells/mL based on count and dilution factors according to the instructions for the hemacytometer.

#### 3.1.3.2. 0.5 McFarland Standard/Optical Density

The optical density at 625 nm (OD$_{625}$) (a wavelength of 530–650 nm is acceptable, as long as all readings are consistent) of a 0.9% saline suspension of the test organism can be compared with either the 0.5 McFarland Standard (a suspension of BaSO$_4$) or the viability count on agar (see Subheading 3.1.3.3.). The OD$_{625}$ of the 0.5 McFarland is equivalent to approx $3 \times 10^6$
Table 3
Comparison of Counting Methods Using *C. albicans* ATCC 90028

<table>
<thead>
<tr>
<th>Attempt</th>
<th>OD&lt;sub&gt;625&lt;/sub&gt; Test organism (–OD&lt;sub&gt;625&lt;/sub&gt; saline)</th>
<th>OD&lt;sub&gt;625&lt;/sub&gt; 0.5 McFarland (–OD&lt;sub&gt;625&lt;/sub&gt; H&lt;sub&gt;2&lt;/sub&gt;SO&lt;sub&gt;4&lt;/sub&gt;)</th>
<th>0.5 McFarland CFU/mL estimate</th>
<th>Viability counts&lt;sup&gt;a&lt;/sup&gt; (CFU/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 1</td>
<td>0.243</td>
<td>0.0265</td>
<td>2.75 × 10&lt;sup&gt;7&lt;/sup&gt;</td>
<td>1.99 × 10&lt;sup&gt;7&lt;/sup&gt;</td>
</tr>
<tr>
<td>Day 2</td>
<td>0.0715</td>
<td>0.027</td>
<td>7.94 × 10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>3.41 × 10&lt;sup&gt;6&lt;/sup&gt;</td>
</tr>
<tr>
<td>Day 3</td>
<td>0.164</td>
<td>0.0275</td>
<td>1.79 × 10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>5.10 × 10&lt;sup&gt;6&lt;/sup&gt;</td>
</tr>
<tr>
<td>Day 4</td>
<td>0.0125</td>
<td>0.0285</td>
<td>1.32 × 10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>1.19 × 10&lt;sup&gt;6&lt;/sup&gt;</td>
</tr>
<tr>
<td>Day 5</td>
<td>0.052</td>
<td>0.0345</td>
<td>4.52 × 10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>4.23 × 10&lt;sup&gt;6&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup>Counts have considered any dilution factors used to obtain the colony-forming unit/mL of the undiluted saline suspension OD<sub>625</sub>, optical density at 625 nm; CFU, colony-forming unit.
Fig. 3. Comparison of inoculum counting methods using *Candida albicans* ATCC 90028. Optical density and colony-forming unit/mL estimation using the 0.5 McFarland relationship is compared with resulting colony counts obtained on agar (viability counting). OD, optical density; CFU, colony-forming unit.

CFUs/mL of yeast (*Candida* and *Cryptococcus*) and *Aspergillus* conidia. The 0.5 McFarland also assumes consistent viability of the cells.

1. Resuspend colonies in 0.9% saline (*see Note 5*).
2. Prepare a 0.5 McFarland standard.
3. In duplicate, add 100 µL of the cell suspension, 0.5 McFarland suspension, H$_2$SO$_4$, and 0.9% saline to the wells of a 96-well microplate.
4. Read the plate at 625 nm.
5. Use the following equation to calculate the CFU/mL of the cell suspension:

\[
\frac{\left( \text{Average OD}_{\text{test organism}} - \text{Average OD}_{\text{saline}} \right) \times 3,000,000}{\left( \text{Average OD}_{0.5 \text{ McFarland}} - \text{Average OD}_{\text{H}_2\text{SO}_4} \right)} = \text{CFU/mL test organism}
\]

3.1.3.3. Viability Counting

This method provides the ultimate measure of “potency” of the test inoculum affording a true CFU/mL measurement and can be compared with the two methods mentioned previously.
1. Resuspend colonies in 0.9% saline (see Note 5).
2. Dilute the cell suspension appropriately to afford (based on a rough estimate of the OD_{625}) approx 1000 CFU/mL.
3. Apply 50 µL of the diluted suspension to the surface of an SD agar plate (at least one replicate is recommended), spread with a cell spreader, and allow it to dry under a hood (to prevent colonies from running together).
4. Incubate at 35°C for 48 h for Candida, 35°C for 72 h for Cryptococcus, or 30°C for 72 h for Aspergillus.
5. Count the number of colonies and determine the CFU/mL of the original test suspension taking into account any dilution factors.

3.1.4. Broth Selection and Incubation

Medium composition and pH are also known to affect the activity of samples (45–47). We also have observed acidification of the medium with growth of *C. albicans* in unbuffered SD broth, possibly changing the ionization state of compounds in the test sample, and thus the observed activity. The desirable broth is one that allows consistent growth and does not interfere with OD readings. The addition of a buffer, such as MOPS, to the chosen broth will allow the pH to remain stable throughout incubation. Media types include rich (unlimited nutrient pool) and minimal media (restricted nutrients) in which the constituents and their concentrations may be known (synthetic, defined) or unknown (undefined). Theoretically, it is possible that rich media may be more likely to mask the antifungal effects of extract constituents by supplying a broader range of nutrients in undefined ingredients, such as yeast extract, that may counteract the activity of antifungal compounds. Figure 2 illustrates the effect broth type and pH can have on the activity of crude extracts (Fig. 2A) and a pure compound (Fig. 2B) with an ionizable carboxylic acid group.

NCCLS recommends using a completely synthetic medium such as RPMI-1640 buffered with MOPS at pH 7.0 (supplemented with dextrose at 2% [48]) to eliminate the variability of undefined constituents. Moreover, when using genetically altered strains or specialized assays (see Subheadings 3.1.2. and 3.3.), the choice of medium may become more significant. For example, when growing genetically altered strains, it may be necessary to supplement the medium with certain amino acids and/or nitrogenous bases if the cells contain auxotrophic markers for selection. With mode of action assays (see Subheading 3.3.), it may be necessary to confirm that the supplement being added to the medium is not already included in the medium constituents (e.g., yeast extract, a water-soluble extract of yeast that contains amino acids, vitamins, and minerals). Furthermore, certain gene products may be suppressed by medium conditions, such as pH (49). Therefore, a defined medium with appropriate supplements may be desired in these situations. One may also choose a broth that produces a certain phenotypic response, such as induction of mycelia formation (50).
3.1.5. End Point Determination

The standard end point for general antifungal screening of fungi is either one of OD or visual observation by comparison to negative (growth in solvent/diluent only), positive (growth in the presence of antifungal agent affording 0% growth), and blank (sterile medium only) controls. Activity can be assigned as the minimum inhibitory concentration ([MIC], lowest test concentration that allows no detectable growth), or various intermediate inhibitory concentrations (i.e., IC\textsubscript{50}, or the concentration that affords 50% growth). If measuring OD, it may be helpful to cover assay plates with clear sealers (i.e., SealPlate film; Sigma, St. Louis, MO) after incubation and gently shake plates to afford a consistent suspension. When working with natural products, often color and precipitation due to the extract can mask growth of the organism (see Note 2). An initial OD reading prior to incubation can be subtracted from final OD readings, and only the relative increase in turbidity (which is assumed to result from only microbe growth) can be used when calculating activity:

\[
100 \times \frac{(\text{OD Final}_{\text{test sample}} - \text{OD Initial}_{\text{test sample}}) - (\text{OD Final}_{\text{blank}} - \text{OD Initial}_{\text{blank}})}{(\text{OD Final}_{\text{negative}} - \text{OD Initial}_{\text{negative}}) - (\text{OD Final}_{\text{blank}} - \text{OD Initial}_{\text{blank}})} = \% \text{ Growth}
\]

Other methods for monitoring growth of fungi include the oxygen biosensor method (51), tetrazolium salts (52), and Alamar Blue\textsuperscript{TM} reduction (53); these may avoid/minimize the effects of sample interference (see Note 2). Using colorimetric or oxygen sensor methods may also allow the reduction of incubation times; OD changes may not be sufficient unless prolonged incubation times are used. Oscillation of the assay plates to enhance growth can also be considered (54,55). With any method, the overall difference in positive and negative controls should be large enough, and deviation within each should be small enough, to reliably assign activity to test samples. A convenient approach for gauging assay performance is suggested by Zhang et al. (54):

\[
1 - \frac{3 \times \sigma_{\text{positive control}} + 3 \times \sigma_{\text{negative control}}}{\text{ABS} | \mu_{\text{positive control}} - \mu_{\text{negative control}} |} = Z'.
\]

Where $\sigma$ = standard deviation; $\mu$ = average; ABS = absolute value; a value for $Z'$ of 0.5 – 1 indicates excellent assay quality.

Therefore, using the NCCLS methods as a starting point, an antifungal screening laboratory can personalize a microplate-based screening protocol using the above variables. We have employed a modified version of the NCCLS methods for the antimicrobial screening program at the National Center for Natural Products Research (57–59).
3.2. Bioautography

Bioautography is a simple method to assign activity to compounds in crude preparations often found in a natural products laboratory using a combination of bioassay and TLC (for TLC methods, see ref. 16). Two general methods are available: agar diffusion (60) and direct TLC (61).

3.2.1. Agar Diffusion Bioautography

The following example employs C. albicans ATCC 90028, but any other fungus may be used.

1. A suitable TLC solvent system is chosen for optimum separation of constituents in an active extract or fraction (detected by a microplate screen). Spots visualized with ultraviolet light or spray reagents are circled with a pencil. Prior to bioautography, the TLC plate must be completely free of solvent. (Note that many spray reagents, such as sulfuric acid, can destroy compounds on the TLC. A reference TLC may be used in this case.)
2. The Candida inoculum (prepared using methods in Subheading 3.1.3. to afford $1 \times 10^6$ CFU/mL) in SD broth is swabbed onto a 50-mL SD agar plate and allowed to dry for 15–30 min.
3. The marked TLC is placed face down directly onto the surface of the agar for 30 min and removed.
4. The agar plates are incubated at 35°C for 24 h, and any zones of growth inhibition are noted and compared with the TLC for isolation of active constituents.

3.2.2. Direct TLC Bioautography

Nonpathogenic fungi may be sprayed directly onto a TLC plate using a device such as a chromatographic sprayer provided that the organism will grow adequately on the TLC medium (silica gel is compatible). However, Candida, Cryptococcus, etc. should be applied using an autoclavable roller device (draped with sterile filter or chromatography paper) under a biological safety cabinet. Visualization of growth with tetrazolium salts (62) may be necessary depending upon the inherent color of the organism. A two-dimensional version of direct TLC bioautography allows improved resolution of the components of the extract (63).

1. TLCs plates are prepared as in Subheading 3.2.1.
2. The Candida inoculum (prepared using methods in Subheading 3.1.3. to afford $1 \times 10^6$ CFU/mL) is rolled directly onto the TLC plate.
3. The TLC plate is placed within an empty large Petri dish containing sterilized wet paper towels or filter paper to increase the humidity, and incubated at 35°C for 24 h.
4. Using a chromatographic sprayer/atomizer, a solution of a tetrazolium dye (e.g., 0.25% aq. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide) is applied to the TLC plate, and the plate is incubated for 4–6 h.
Fig. 4. Synergy of two compounds (A and B) isolated from *Cassia* sp. (57). FIC, fractional inhibitory concentration (see Note 7).

5. A fixing solution of 70% EtOH is sprayed onto the TLC plate, and any zones of inhibition are detected by a lack of color.

Bioautography offers a rapid and convenient approach to identify antifungal constituents of a crude extract or fraction for facilitating bioassay-directed isolation, and requires only microgram quantities. However, these techniques assume that a. the constituents diffuse into the agar to afford sufficient quantities to exert antifungal activity; b. the original activity seen in the crude extract or fraction is not caused by synergy between several compounds, which alone are less potent or inactive (see Note 6 [64–75]; Fig. 4); and c. the compounds do not decompose during TLC. The activity of any constituents, once isolated, should be confirmed using a microplate assay.

3.3. Mode of Action Studies

Mode of action studies (vs mechanism of action studies in which an isolated target is assayed), can suggest the general biological process perturbed by an antifungal agent and whether that action is fungicidal or fungistatic. Basic types of mode of action whole-cell experiments include the following:

1. Detection of fungicidal vs fungistatic actions.
2. Protection/rescue assays.
3. Drug combination/interaction assays.
3.3.1. Fungicidal vs Fungistatic Actions

A fungicidal agent kills cells at concentrations similar to its MIC. Conversely, a fungistatic compound, although having a potent MIC or IC₅₀, may be unable to kill fungal cells. For example, fluconazole successfully inhibits the growth of *C. albicans* at fairly low-test concentrations but only kills cells at concentrations greater than 100× its IC₅₀. Conversely, the β-glucan synthase inhibitors are potently fungicidal. Therefore, a drug being “static” or “cidal” may give clues about the general biological process affected. The minimum fungicidal concentration (MFC) can then be compared with the MIC to gage the extent of fungicidal activity.

1. Using methods mentioned in Subheading 3.1., evaluate the antifungal activity of a pure compound of interest in addition to a positive control (i.e., amphotericin B).
2. After incubation and before sealing the assay plate, transfer to agar 5 µL from each well showing no detectable growth and allow to dry under a hood (to prevent drops from running together).
3. Incubate agar plates at 35°C for 48 h for *Candida*, 35°C for 72 h for *Cryptococcus*, or 30°C for 72 h for *Aspergillus*.
4. Determine the MFC by recording the lowest test concentration in which no growth on agar is seen. (Each laboratory may have its own criteria, allowing no growth or one to two colonies at each concentration as the cut-off.)

3.3.2. Protection/Rescue and Combination Studies

Examples of protection/rescue assays include protection of cell integrity in the presence of both a cell wall synthesis inhibitor and an osmoprotectant (76), and counteraction of the effect of ergosterol (77) or sphingolipid (78) synthesis inhibitors via rescuing with appropriate biosynthetic intermediates. Drug interaction/combination assays include studies in which a sample may be relatively inactive alone in vitro, but only in combination becomes significantly active. An example of this type of assay includes inhibition of azole resistance in yeast by disabling efflux pumps (79,80), and a similar approach has been used in resistant bacteria (81,82). The desired effect of both assays is resistance of the sample-treated organism in the presence of the protecting substance/biosynthetic intermediate, or increased susceptibility in the presence of a substance that enhances a compound’s activity.

3.3.2.1. Checkerboard Assays

The i-checkerboard assay is a method that allows a “two-dimensional” analysis of the interaction of two agents on the microbe in question. The following example (see Fig. 5) assesses extracts for their ability to enhance the activity of fluconazole (FLU). The *S. cerevisiae* strains used in this assay con-
tain plasmids overexpressing \textit{C. albicans} efflux pumps (83–85), which are known to cause drug treatment failure (86).

1. \textit{S. cerevisiae} strains containing the CDR1 and MDR1 \textit{C. albicans} efflux pumps along with the null pump (plasmid only; to detect pump-independent effects) are grown on YNB selection agar (-uracil/-tryptophan) at 30°C before the assay to afford single colonies.
2. FLU and the test sample (TS) in question are dissolved in DMSO and serially-diluted using 0.9% saline in a 96-well microplate. The FLU dilutions are made along the x-axis (horizontally) and the TS dilutions along the y-axis (vertically). The last column (or row) contains saline only.
3. Both the FLU and TS dilutions are transferred to a 96-well flat bottom microplate (10 µL each) affording 12 columns of identical TS dilutions and eight rows of identical FLU dilutions. All concentration permutations are afforded and negative (well H11) and blank (well H12) controls are included on the assay plate.
4. The \textit{S. cerevisiae} inoculum (prepared in YPD using methods described in Subheading 3.1.3. to afford 1 × 10⁴ CFU/mL) is added (180 µL) to all wells excluding the blank, in which 180 µL YPD only is added (Alamar Blue™ affording a final concentration of 5% may be supplemented to the medium for a colorimetric assay).
5. The assay plates are read at 625 nm (or 544 ex/590 em if using Alamar Blue™) prior to and after incubation at 30°C for 48 h.

In order to determine the significance of the observed activity, methods such as the fractional inhibitory concentration (FIC) (87), isobolograms (88,89; see Note 7), and volume of synergy (90) may be used.
4. Notes

1. It is known that screening plant extracts in isolated enzyme assays results in many false positives due to the presence of tannins and other compounds such as aldehydes (28,29). Tannins possess the ability to nonselectively precipitate proteins, thus leading to false positives in assays of enzyme inhibition. We have chosen to identify extracts high in tannins and either “de-tanninize” or deselect them. Several methods exist to eliminate tannins including polyamide chromatography, and polyvinylpyrrolidone or gelatin precipitation (28,30). In Table 1, the effect of polyamide chromatography on the activity of a crude plant extract in two isolated enzyme assays (glucosyltransferase and protease inhibition) is illustrated. For tannin detection, we have adapted a gelatin/FeCl₃ test to a 96-well microplate format to afford the “Tannin Index.” High tannin indices are consistent with high tannin content. Figure 1A shows the comparison between tannin indices and % inhibition in a protease assay. Figure 1B illustrates the effect of tannic acid at varying concentrations on the precipitation of gelatin and the reaction of phenolic hydroxyls with FeCl₃. Compounds other than tannins may possess either of these qualities, but tannins usually possess both.

Analysis for tannins:

1. 10 µL of a 20 mg/mL extract in DMSO is added to the wells of a 96-well flat bottom microplate in triplicate.
2. 10 µL of tannic acid concentrations (20, 10, and 5 mg/mL in DMSO) is added to the microplate with three replicates/tannic acid concentration.
3. 10 µL of DMSO is added in triplicate as a control.
4. 200 µL of a 10% aq. NaCl solution is added to replicates #1.
5. 200 µL of a 10%/1% aq. NaCl/gelatin solution is added to replicates #2.
6. 200 µL of a 0.2% FeCl₃ solution is added to replicates #3.
7. The plate is read at 625 nm and the following calculations are used to afford a “Tannin Index.”

\[
\frac{(OD_{Sample_{NaCl/gelatin}} - OD_{Sample_{NaCl}}) + (OD_{Sample_{FeCl₃}} - OD_{DMSO_{FeCl₃}})}{2} = \text{Tannin Index}
\]

2. More test concentrations may be reserved for increasing purity of the sample (e.g., one test concentration for primary screening of crude extracts [to afford a percent inhibition] to 6–12 concentrations for pure compounds [for IC₅₀ and MIC determination]). In Fig. 6, the sample, tested at one concentration of 200 µg/mL in a primary screen, would have been considered inactive and overlooked with 0% inhibition vs C. neoformans, in which OD readings determined the endpoint. When tested against the bacterium Mycobacterium intracellulare using an Alamar Blue™ method independent of OD, no interference at high sample concentration was observed. The set of test concentrations used will ultimately affect the hit rate (affecting the number of samples subjected to bioassay-directed fractionation), and higher test concentrations generally produce higher sample interference (affecting the number of false negatives and positives; see Note 3).
Fig. 6. Comparison of optical density vs Alamar Blue™ end point illustrating a false-negative reaction of an extract from a marine sponge. OD, optical density; SD, Sabouraud dextrose.

Generally, screening crude extracts at final concentrations of greater than 100 \( \mu g/mL \) generates inordinately high hit rates. It is recommended that prior to bioassay-directed fractionation of crude extracts, rough dose response estimates (e.g., three test concentrations) of hits from a primary screen are conducted to confirm activity in order to prevent pursuing false positives (see Notes 1 and 3).

3. Analysis of crude extracts in bioassay systems can be problematic because of interference of color, lack of water solubility, and presence of nuisance compounds. Lack of water solubility may be ameliorated by adding PEG or a similar solubilizing/suspending agent to the medium or sample (19,20). OD readings taken before incubation also may serve as background correction, but precipitation of sample constituents with incubation may mask activity. In Fig. 6, lowering the test concentration decreased the interference of the sample (an extract from a marine sponge) to reveal its potent activity, an illustration of a false-negative result.

4. A variety of diluents may be used to dilute samples including assay medium, 0.9% saline, or DMSO. However, the ratio of the diluted sample:inoculum should be such that the final solvent concentration is no more that 1% and the inoculum is not diluted significantly with a medium not conducive to growth. In order to minimize sample precipitation and keep solvent concentration consistent, DMSO may be used for serial dilutions, but samples must be diluted further in assay
medium or 0.9% saline prior to transfer to the assay plates. For example, samples may be serially diluted in DMSO, diluted 1:5 with 0.9% saline, and transferred to assay plates. A ratio of 1:20 diluted sample:inoculum (10 µL sample:190 µL inoculum) affords a final DMSO concentration of 1%.

5. For each set of counts, cell suspensions should be prepared by resuspending five colonies of approx 1 mm in diameter (obtained from freshly prepared agar plates of frozen stocks) in 0.9% saline. For *Aspergillus* cultures, conidial suspensions are prepared by flooding the surface of growth on agar with 0.9% saline and gently scraping to remove spores using an inoculating loop. The resulting *Aspergillus* suspension should either be allowed to settle or filtered through sterilized Miracloth to remove mycelial fragments. The resultant suspensions are used in the counting methods in Subheading 3.1.3. The wells of a microplate are sufficient for determining the ODs of the 0.5 McFarland standard and test organism suspensions. Variously aged cultures on agar may also be tested to determine how long similar viability is retained. Once a consistent relationship is established between a hemacytometer count, the 0.5 McFarland estimate or OD to CFUs obtained on agar, any of these methods may be used prior to each assay in lieu of viability counting (see Table 3 and Fig. 3). However, several freeze/thaw cycles of frozen stocks may decrease the viability of a culture, and (especially after obtaining a new lot of an organism), viability counting should be conducted periodically to ensure that proper CFU/mL measurements are retained.

6. The bottleneck in discovery of antimicrobial compounds from natural products is usually isolation and purification (64). Therefore, reisolation of the same active constituents should be minimized by using literature searches and spectrometric/physicochemical screening methods simultaneously with bioassays to deselect extracts known to produce antifungal compounds (65–67). Conversely, collection (location, time or season) and extraction strategies, along with ethnobotanical information, may be used to help ensure selection of active extracts (67–74). Criteria, such as lack of cytotoxicity of extracts against mammalian cells (75), potency and selectivity, ease of recollection, and lack of presence in the literature, may be used to prioritize samples to pursue (12). Loss of activity with purification is commonly encountered and may be the result of several factors including decomposition or synergy of several different compounds (see Fig. 4).

7. The FIC is calculated using the following formula (FIC: ≤0.5 = synergistic; 0.51–1 = additive; 1.1–2 = indifferent; >2 = antagonistic):

$$\frac{IC_{50} \text{ or MIC TS in combination with FLU}}{IC_{50} \text{ or MIC TS alone}} + \frac{IC_{50} \text{ or MIC FLU in combination with TS}}{IC_{50} \text{ or MIC FLU alone}} = FIC$$

However, by the nature of the checkerboard assay, several choices of concentration combinations can be used to calculate the FIC (see Fig. 7A). The selected concentration of FLU or the TS should be high enough that an effect is seen in combination, yet low enough that any inherent antifungal activity of FLU or TS alone is minimal or nonexistent. An alternate method for gauging synergy or
Fig. 7. Methods for analyzing checkerboard assays. (A) Dose–response curves of the test sample (TS) and fluconazole (FLU) alone and in combination at optimum concentrations (see Subheading 3.3.2.1.). The fractional inhibitory concentration (FIC) is calculated using the selected end point (inhibitory concentration that affords 50% growth [IC$_{50}$]). (B) Isobologram of the same 2-drug experiment. The additivity line connects the IC$_{50}$ of the TS and FLU alone and represents all theoretical combinations that produce the same end point. However, the IC$_{50}$ of the TS was greater than the highest test concentration of 100 µg/mL (indicated by an arrow). The additivity line is therefore drawn to afford a more conservative estimate of synergy.
antagonism is the isobologram (see Fig. 7B); however, the same caveats for FIC determination exist. The isobologram assumes that varying ratios of FLU and TS (i.e., 1/2 IC$_{50}$ FLU + 1/2 IC$_{50}$ TS) can elicit the same response (IC$_{50}$) as with FLU or TS alone (additivity line). Any significant deviation below or above this line suggests synergy or antagonism, respectively. However, standard deviations of both the additivity line and end point (IC$_{50}$) of each compound should be taken into consideration.

Acknowledgments

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References


Use of an Animal Model of Disseminated Candidiasis in the Evaluation of Antifungal Therapy

David Andes

Summary

Animal models have been helpful in assessing a drug’s potential application to treatment of humans. These controlled experiments allow description of the impact of a wide range of important treatment variables, including drug dose or concentration, dosing interval, pathogen, and host immune state. Animal models of mycoses are designed to address particular therapeutic questions. Some models are designed to screen multiple compounds in a rapid manner. Other models are selected to more closely mimic clinical infections. The following chapter will be limited to the description of a murine model of disseminated candidiasis in which in vitro results, pharmacokinetic properties, and a microbiological in vivo end point is used in the assessment of a wide variety of antifungal compounds.

Key Words: Candida; antifungals; animal model; pharmacokinetics; pharmacodynamics; drug development; in vitro susceptibility; drug resistance.

1. Introduction

Numerous animal models have been useful in the development of antifungal drugs (1–12). The following chapter describes the use of a neutropenic murine model of disseminated candidiasis to compare the in vivo potency of antifungals with the same drug class, determine the most appropriate dosing interval and dose level choice, and provide information useful for understanding the implications of in vitro susceptibility test results (13–16).

The antifungal drug treatment analysis that allows one to undertake this extrapolation from a murine infection model to infections in other animal species including humans is termed pharmacokinetics and pharmacodynamics (17,18). Studies begin with a detailed analysis of antifungal pharmacokinetics, which includes protein binding, and consideration of three pharmacodynamic parameters (the time that serum levels exceed the minimum inhibitory concen-
tration [MIC]; the area-under-the-concentration curve [AUC] in relation to the MIC, or AUC/MIC; and the peak level in serum in relation to the MIC, or peak/MIC).

Initial treatment studies begin with escalating single dose time-kill studies that determine the impact of drug concentration on the rate and extent of killing and the presence or absence of antimicrobial effects that persist after brief drug exposure or postantibiotic effects (19). Next, researchers performing treatment studies use a wide range of dosing regimens to determine the impact of both dose level and dosing interval on outcome, which is useful in determining which dosing strategy or pharmacodynamic parameter is better associated with drug efficacy (18,20). Finally, dose-ranging treatment studies are performed with a large number of organisms with widely varying susceptibility in vitro (17,18). These studies are designed to define the pharmacodynamic parameter magnitude predictive of treatment efficacy. With knowledge of this pharmacodynamic target, one can begin to consider the impact of drug dosing in humans and the impact of drug resistance for the development of optimal dosing and the in vitro susceptibility break-point guidelines (16,20–25).

2. Materials

2.1. Organisms

2. Sabouraud dextrose (SD) agar.
4. 96-Well microtiter plates.
5. 37°C Incubator.
6. –70°C Freezer.
7. Spectrophotometer.
8. Hemocytometer.
9. Microscope (40× magnification).
10. Yeast nitrogen base (yeast peptone dextrose [YPD]) + 20% glycerol.
11. 0.9% NaCl.

2.2. Antifungal Drugs

1. Metler balance.
2. 0.9% NaCl.

2.3. Animal Infection Model

1. Animal care facility.
2. Swiss ICR Mice 6–8 wk of age.
3. 1-mL Syringe (tuberculin).
4. 3-mL Syringe.
5. Cyclophosphamide.
6. 26- or 27-gage needles.
7. Tissue homogenizer.
8. 70% Ethanol.
9. 5- and 10-mL plastic or glass tubes.
10. Centrifuge (microcentrifuge and macrocentrifuge rotors).
11. CO₂ tank/chamber.
12. Antifungal drug(s).

2.4. Drug Studies

1. 26- or 27-gage needles.
2. 1-mL Syringe.
3. Capillary tubes (heparinized).
4. Halothane.
5. Bell jar.
6. Fume hood.
7. Capillary tube centrifuge.
8. Millipore ultracentrifugation filters.
9. Microbiological assay organisms.
11. SD agar.
12. Statistical and pharmacokinetic analysis software.

3. Methods

3.1. Organisms

3.1.1. Selection of Isolates

A single clinical isolate of *C. albicans* is used for studies to describe the antifungal drug killing characteristics over time. A larger number of clinical *C. albicans* isolates are used to determine the in vivo potency of the antifungal drug relative to organism in vitro susceptibility or the MIC. We consider two factors in choosing this group of organisms. Isolates are chosen to include a wide range of organism MICs (the range of MICs identified in large surveillance databases [26]). Furthermore, when available, we attempt to utilize organisms with reduced antifungal drug susceptibility for which the resistance mechanism(s) is known in order to determine the impact of these mechanisms on in vivo outcomes (13,14,16). In addition, we attempt to limit variation in organism virulence in the infection model to allow the antifungal drug potency and drug-dosing regimen to be the primary dependent variables in these studies.

3.1.2. Organism Storage

Organisms are stored in YPD + 20% glycerol at –70°C. Twenty-four hours before the study, organisms are subcultured on a SD agar plate or slant at 37°C.
3.1.3. Preparation of Inoculum

1. Organisms from stock isolates stored at –70°C are streaked onto SD agar and incubated at 37°C for 24 h.
2. After subculture, three to five individual colonies 2–5 mm in diameter are sus- pend ed in 5 mL of 0.9% NaCl.
3. The optical density (OD) of this inoculum is adjusted to 0.65 transmittance at 530 nm by dilution in 0.9% NaCl (see Note 1 [12,27]).
4. The number of blastoconidia in the inoculum are further estimated by means of hemocytometer counts and then verified by plating serial 10-fold dilutions on SD agar plates.

3.1.4. In Vivo Isolate Virulence

We measure the growth of organisms in the kidneys of untreated mice over the planned treatment period (see Note 2 [1,12–15,28–34]). The limit of organism growth in kidneys for inclusion varies depending upon the planned study duration. For a 24-h treatment period, we anticipate organism growth ranging from 2 to 3.5 log\(_{10}\) colony-forming unit (CFU)/kidneys. For longer treatment periods (72–96 h), we expect growth ranging from 3.5 to 5.5 log\(_{10}\) CFU/kidneys. We eliminate organisms from consideration that are determined to be less virulent than others in the group.

3.1.5. In Vitro Susceptibility Testing

1. MICs are determined using the National Committee for Clinical Laboratory Standards (NCCLS M27-A) method (35).
2. Determinations are performed in duplicate on at least two separate occasions.
3. Final results are expressed as the geometric mean of these results.

3.2. Antifungal Drugs

3.2.1. Drug Storage and Preparation

1. When possible, antifungal compounds are obtained from the respective pharmaceutical company in powder form.
2. The drugs are stored in accordance with respective recommendations to ensure stability.
3. Drug concentrations are prepared based on drug powder percent purity.
4. The stock drug solution concentration is prepared on the day of study and appropriate dilutions prepared with an acceptable physiological diluent (e.g., 0.9% NaCl [36]).

3.3. Animal Infection Model

3.3.1. Animals and Animal Care

1. Six-week-old, specific pathogen-free female Swiss ICR (CD1) mice can be purchased from Harlan Sprague–Dawley (Indianapolis, IN; see Note 3 [3–6,8–12,27,28,37–40]).
2. Before the study, the animals should be housed in groups of five per cage and allowed to acclimate 7 d before the initiation of experiments.
3. Mice should be allowed free access to food and water.
4. At the time of study, the animals should weigh 23–27 g.
5. All of our animal studies were approved by our local animal research committee and are in accordance with both the National Institutes of Health Animal Care and American Association for Accreditation of Laboratory Animal Care guidelines.

### 3.3.2. Immunosuppression

A profound state of granulocytopenia (<100/mm³) is produced in animals to maximize the growth of *Candida* in mice (*see Note 4*). This immunocompromised state can be achieved by administration of cyclophosphamide prior to infection. Cyclophosphamide is administered via intraperitoneal injection 4 d (150 mg/kg) and 1 d (100 mg/kg) before the day of infection. This regimen will produce a neutropenic state for 5 d in this animal species. The degree and duration of neutropenia is confirmed by monitoring neutrophil counts of peripheral blood smears with a hemocytometer daily throughout the duration of study.

### 3.3.3. Infection of Animals

1. Neutropenic mice are placed head first into a mouse restraint device with the tail extending freely hanging through the other end (*36*).
2. The tail is immersed in warm (50°C) water bath for 5–10 s to dilate the veins.
3. Using a (1 mL) tuberculin syringe with either a 26- or 27-gage needle the inoculum (0.1 mL of a suspension of 10⁵–⁷ CFU/mL *Candida* blastospores) is injected via the lateral tail vein (*see Note 1*).
4. Animals should be monitored at least every 6 h after infection for the remainder of the study period.
5. Any animals that demonstrate difficulty getting to food or water should be euthanized immediately.

### 3.4. Drug Studies

#### 3.4.1. Pharmacokinetics

1. Serum pharmacokinetic study is critical for the design of dosing regimens for the subsequent treatment experiments (*see Note 5*). The study of a minimum of three escalating dose levels is necessary to provide information regarding the linearity of kinetics and for subsequent estimation of kinetics for antifungal treatment levels where actual kinetic studies were not performed (*36*). The choice of actual dose levels should include the extremes of dose levels anticipated in treatment studies. Often, the lower limit of drug assay detection will determine the lowest dose one is able to accurately examine.
2. Kinetic studies are performed in immunocompromised, infected mice in order to mimic the infection model conditions.

3. A minimum of six sampling time points is necessary to adequately estimate both the elimination half-life and the AUC. We perform primarily single dose studies in groups of six mice per dose level (see Tables 1 and 2). The sampling times after drug administration most often are based on previously published data with similar compounds. When this is unavailable, one must rely on empiricism.

4. Blood from groups of three mice is sampled at each sampling time point. At the next time point the second group of three mice is sampled. Thus, any one mouse is sampled only three or four times to minimize the impact of blood loss on drug concentrations.

5. Before sampling, the animals are anesthetized with a few drops of halothane in a bell jar.

6. Blood (50 µL per time point) is collected in heparinized capillary tubes (Fisher Scientific, Pittsburgh, PA) from the orbital venous plexus (36). The volume of blood collected from any individual animal is less than 5% of the total blood volume.

7. The tubes are immediately centrifuged in a capillary centrifuge (model MB centrifuge, International Equipment Co.) at 10,000 g for 4–5 min, the serum is removed with a micropipet, placed in wells of a 96-well microtiter plate and frozen at −80°C until drug assay.

8. Our laboratory uses either microbiological or high-performance liquid chromatography (HPLC) methods to determine drug concentrations (see Note 6 [41]). Discussion of specific methodology is beyond the scope of this chapter. Assay of six to eight standards of drug-spiked serum (twofold escalating concentrations) to create a standard curve is similarly assayed. The lower limit of detection for the assay is defined, and the intra- and interday variation calculated. Pharmacokinetic constants for each drug dose level are calculated using the drug concentrations from the individual mouse samples. The elimination half-life in the postdistributive phase is calculated using a noncompartment model and an unweighted least linear squares methods (42). The AUC is calculated using the linear trapezoidal rule up to the final measured concentration and then extrapolated to infinity (43).

3.4.2. Protein Binding

1. Serum protein binding of anti-infective drugs can reduce antimicrobial activity, restrict tissue distribution, and delay drug elimination (44).

2. We undertake protein-binding studies in the serum of infected, neutropenic mice with each antifungal drug studied using an ultrafiltration method (see Note 7 [44]).

3. At least three drug concentrations are studied. The range of concentrations should reflect the concentrations found in the pharmacokinetic measurements when possible. Often the lowest concentrations one is able to study are limited owing to the lower limit of detection of the assay and high degrees of protein binding with some antifungal compounds.
Table 1
Example of Drug Treatment

<table>
<thead>
<tr>
<th>Dose level/time</th>
<th>Group 1</th>
<th>Group 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 mg/kg 1 h</td>
<td>A</td>
<td>B</td>
</tr>
<tr>
<td></td>
<td>A1</td>
<td>B1</td>
</tr>
<tr>
<td>1 h</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3 h</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6 h</td>
<td>A2</td>
<td>B2</td>
</tr>
<tr>
<td>12 h</td>
<td></td>
<td></td>
</tr>
<tr>
<td>18 h</td>
<td>A3</td>
<td>B3</td>
</tr>
<tr>
<td>24 h</td>
<td></td>
<td></td>
</tr>
<tr>
<td>36 h</td>
<td>A4</td>
<td>B4</td>
</tr>
</tbody>
</table>

4. After the mouse serum is spiked with the antifungal drug, a 1-mL aliquot is filtered by centrifugation through a Millipore YM10 filter (10,000 mw exclusion) device (Millipore Corp, Bedford, MA).
5. The device is centrifuged using a fixed angle rotor at 5000 g for 5 min.
6. Drug concentrations are determined in serum and in ultrafiltrate to determine the degree of protein binding at each of the drug concentrations.

\[
\% \text{ Protein Bound Drug} = \left( \frac{[\text{Serum}] - [\text{Ultrafiltrate}]}{[\text{Serum}]} \right) \times 100
\]

3.4.3. Animal Sample Size Justification

3.4.3.1. Pharmacokinetics and Time-Kill

We have found that the use of two mice per experimental condition (i.e., sampling time, dosing regimen) to be adequate for estimating the underlying variability of replicate observations (see Table 3). The additional benefit of using three or more mice per condition is minimal. Thus, for the study of time course antifungal activity of a single dose of drug, two mice are euthanized at each time point for both treated and untreated control animals.

3.4.3.2. Pharmacokinetic/Pharmacodynamic Studies

In vivo dose–response data are best described by a nonlinear model. We use a nonlinear \( E_{\text{max}} \) model, which allows us to observe data points ranging from no effect to maximal effect (17,18). To fit a three-parameter nonlinear model, a minimum of three distinct data points is needed. To assess lack of model fit, additional data points (doses) are needed. Thus, the use of two or three additional data points (for a total of five or six) provide some ability to detect lack
of fit in the model and make comparison of fitted curves across dosing intervals feasible. Thus, to estimate each dosing relationship with a fixed dosing interval requires 10–12 animals (2 animals for each of five to six doses).

These experiments are further designed to determine which Pharmacokinetic/Pharmacodynamic (PK/PD) parameter (AUC/MIC, peak level/MIC, or T > MIC) is the best predictor of the efficacy of various drug organism combinations (18). There are notable interrelationships among these dosing indices when using a single dosing interval. To make the indices distinct, we require the use of more than a single dosing interval. Most frequently, three or four dosing intervals are needed to provide sufficient variability to make the parameters distinguishable. In the analysis we compare treatment outcomes with untreated control animals both at the start of therapy (two mice) and at the end of therapy (two mice). Thus, each experiment with a different drug–organism combination requires up to 52 total mice (Table 5).

In optimal-dosing studies, we limit our study to the most efficacious single dosing interval. This reduces the number of mice to 16 per drug–organism combination (12 treated and 4 untreated controls) (Table 6).

### 3.4.4. Antifungal Drug Treatment

1. Drug treatment is initiated 2 h after infection (see Note 8). This delay after infection allows the cells to enter the early log phase of growth in the mouse kidneys (12,14).
2. The route of administration is dependent on the absorptive characteristics of the specific antifungal. If possible the subcutaneous route allows for more frequent dosing with minimal risk of damage to the animal. Other routes commonly utilized include intraperitoneal and by oral gavage (36). Intravenous administration rarely is necessary.

3.4.4.1. SINGLE-DOSE TIME-KILL AND POSTANTIFUNGAL EFFECT

1. Three escalating antifungal dose levels are administered to groups of infected animals to determine killing dynamics related to drug concentration and in order to measure antimicrobial effects following drug exposure (see Note 9 [16,19,32]). These later effects are referred to as postantibiotic or postantifungal effects. The rate of killing is determined during an 8- to 12-h period with three to five sampling time points every 1–4 h (see Table 6).

2. To measure the postantibiotic effect (PAE) requires sampling times after it is estimated that serum levels in the mice would have fallen below the MIC of the infecting pathogen. After which, four to five sampling time points over the subsequent 12–24 h are used.

3. Growth of organisms in the mice of drug treated mice is compared with that in untreated control mice. Growth in untreated controls is measured with four to five sampling time points during the course of 12 h.

4. The PAE is calculated by determining the time that it takes for counts from the controls to increase 1 log_{10} CFU/kidneys (C) and subtracting this from the amount of time that it takes organisms from the treated mice to grow 1 log_{10} CFU/kidneys (T) after levels in serum have fallen below the MIC: PAE = T – C (19).

<table>
<thead>
<tr>
<th>Table 4</th>
<th>Animal Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Drug</td>
<td>Dose levels</td>
</tr>
<tr>
<td>Antifungal</td>
<td>5–6</td>
</tr>
<tr>
<td>No drug</td>
<td>1</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td></td>
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</table>

<table>
<thead>
<tr>
<th>Table 5</th>
<th>Animal Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Drug</td>
<td>Dose levels</td>
</tr>
<tr>
<td>Antifungal</td>
<td>5–6</td>
</tr>
<tr>
<td>No drug</td>
<td>1</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td></td>
</tr>
</tbody>
</table>
3.4.4.2. DOSE FRACTIONATION/PHARMACODYNAMIC PARAMETER DETERMINATION

A minimum of a five total drug dose levels are used to describe the entire dose–response relationship, from low doses with minimal efficacy to maximal efficacy (see Note 9 and Table 7).

1. Dose escalation of two- to fourfold between the five dose levels will most often provide this dose range. A minimum of three dosing intervals is required to determine which dosing frequency or PK/PD parameter is most efficacious.

2. The exact dosing fractionation scheme chosen is dependent upon the elimination half-life of the compound (see Note 10). For drugs with elimination half-lives of 8 h or less, the total dose levels can be fractionated into 3, 2, and 1 dose during a 24-h treatment period. For drugs with longer half-lives, similar fractionation can be undertaken during a longer treatment period. The shortest dosing interval should be at least as long as the elimination half-life to minimize drug-accumulation.

3.4.4.2.1. Analysis of the PK/PD Parameter Relationship

1. The relationship between the residual fungal burden in kidney tissue and the PK data are determined by PD modeling \( (17,18) \).
2. Each dosing regimen is considered as a PD parameter value (e.g., $T > \text{MIC}$, AUC/MIC, and peak/MIC).
3. Both total and free drug concentrations are considered.
4. The correlation between efficacy and each of the three PK/PD parameters studied is then determined by nonlinear least-squares multivariate regression analysis (Sigma Stat, Jandel Scientific Software, San Rafael, CA). The coefficient of determination ($R^2$) is used to estimate the percent variance in the change in $\log_{10}$ CFU/kidney over the treatment period for the different dosing regimens that could be attributed to each of the PD parameters.

### 3.4.4.3. Dosing Studies with Multiple Organisms/PD Parameter Magnitude Studies

Dosing ranging studies with the most efficacious dosing interval are undertaken in studies with a larger group of organisms (e.g., 5–20) to determine whether treatment outcomes can be similarly predicted relative to a PD parameter magnitude (Table 4). This is best accomplished by performing experiments using organisms with widely varying MIC values. As in the treatment studies outlined in Subheading 3.4.3.2, five or six dosing levels are studied and the specific dose levels are escalated two- to fourfold for each rise in dose to achieve a range in efficacy from no effect to maximal effect.

#### 3.4.4.3.1. Analysis of the Dose–Response Relationship(s)

Data generated are best described by a nonlinear regression model, specifically we utilize a modification of the Hill equation ($E_{\text{max}}$ dose–response model):

<table>
<thead>
<tr>
<th>Experiment number</th>
<th>Dosing regimen (mg/kg/6 h)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.25</td>
</tr>
<tr>
<td>Organism 1</td>
<td>1a</td>
</tr>
<tr>
<td>Organism 2</td>
<td>2a</td>
</tr>
<tr>
<td>Organism 3</td>
<td>3a</td>
</tr>
<tr>
<td>Organism 4</td>
<td>4a</td>
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<td>8a</td>
</tr>
<tr>
<td>Organism 9</td>
<td>9a</td>
</tr>
<tr>
<td>Organism 10</td>
<td>10a</td>
</tr>
</tbody>
</table>
Andes

\[
\text{Measured Endpoint (CFU)} = \frac{[-\text{Emax}] \times \text{Dosing Variable}^N}{\text{PD}_{50}^N + \text{Dosing Variable}^N}
\]

Where \( \text{E}_{\text{max}} \) = maximal effect, \( N \) = slope of curve, \( \text{PD}_{50} \) = magnitude of dosing variable to produce a 50% effect. We use the coefficient of determination to compare the fit of the data in order to estimate which dosing indices best describe the data. We further compare a variety of dosing efficacy endpoints, such as the \( \text{PD}_{50} \) using a nonparametric tool, analysis of variance (Kruskal–Wallis) with Dunn’s correction for multiple comparisons. We use a statistical software package from SPSS Science, Sigma Stat® (available from http://www.spssscience.com) to aid in these calculations. Statistical significance is defined as a two-sided \( p < 0.05 \). In addition to determination of the \( \text{PD}_{50} \), it often is useful to estimate a variety of other treatment endpoints including the dose necessary to produce no net change in growth relative to the start of therapy or the static dose, the dose necessary for other degrees of the maximal effect (\( \text{ED}_{25}, \text{ED}_{75} \)), or the dose necessary for absolute end points, such as a 1 or 2 \( \log_{10} \) reduction in organism burden relative to start of therapy.

3.4.5. End Point Measurement

Antifungal efficacy is assessed by comparison of the microbiological clearance of \textit{Candida} from kidneys in treated vs untreated control mice (see Notes 11 and 12 [6,21,23,24,27,29,31,33]). Measurement of organism burden in the kidneys of mice has provided a strong in vitro/in vivo correlation of the therapeutic response to a number of antifungal drugs (1,11,13,15,17,21,23).

1. At the end of therapy, animals are euthanized by \( \text{CO}_2 \) asphyxiation.
2. Both kidneys are aseptically removed and placed in 3.4 mL of sterile cold 0.9% NaCl (an initial 1–10 dilution based on weight of kidneys).
4. The kidney tissue is then homogenized using a Polytron homogenizer for 20 s. After homogenization of each tube, the grinder bit is washed with 70% ethanol followed by fresh tap water. This method prevents significant organism carryover from sample to sample.
5. Each tissue homogenate is then serially diluted 10-fold from 10 to \( 10^{-6} \) in sterile 0.9% NaCl.
6. The dilutions are plated in duplicate on SD agar plates for determination of viable fungal colony counts after incubation at 37°C for 24 h.
7. Counts are expressed as the mean ± standard deviation \( \log_{10} \) CFU/kidneys. Previous studies with this series of dilutions have demonstrated that the method is sensitive to detection of 100 CFU/kidneys.
3.4.6. Potential for Drug Effect From Drug Carry-Over

Occasionally with higher drug doses, remaining antifungal drug concentrations in the tissue homogenate can exert a drug effect on organisms after plating. This is usually easily detected because of the plating of a 10,000-fold range of dilutions. One should observe a 10-fold change in organism counts from dilution to dilution. If one observes a larger number of organisms with dilutions or if one observes significant variation in colony size within a quadrant this may be owing to drug effect.

1. In this situation, the original homogenate is pelleted at 5000 g for 10 min. The supernatant is removed and the pellet washed twice with 0.9% NaCl. The final cell pellet is then resuspended to the original homogenate volume and dilutions and plating are repeated.
2. If the counts from the washed cells have higher counts, this is attributable to the drug effect.
3. In this situation, homogenates with this and higher drug doses should be washed before plating to minimize the drug effect.

4. Notes

1. The infecting inoculum is chosen based on the desired duration of study. The higher the inoculum the shorter the duration of survival of untreated or saline treated control animals (12,27). For studies of 72–96 h, an inoculum of $10^5$ CFU/mL is used. For studies of 24 h, an inoculum of $10^6$ is used.
2. By convention many studies use animal survival following infection as a measure of strain virulence (27–29). We determine relative growth of strains in the kidneys as a measure of virulence because the primary end point of our studies is change in organism burden in the kidneys (1,12–15,30). If one studies organisms with lower virulence in the infection model one often finds that a lower drug dose is needed for efficacy than when one is examining outcome against a more virulent pathogen. To determine the impact of the treatment regimen and not the organism virulence, we try to study organisms with similar virulence. Similarly, if one wishes to examine antifungal outcomes with other Candida spp., we would suggest determining the relative virulence of organisms before the treatment study (6,31–34). In general, nonalbicans species are less virulent. Among the nonalbicans species, tropicalis is the most virulent. In screening large numbers of isolates we have identified virulent Candida krusei. However, we have not found either Candida glabrata or Candida parapsilosis isolates that are virulent enough to allow comparison of treatment outcomes relative to infection with more virulent species.
3. A number of rodent species and rabbits have been commonly used in candidiasis models (3–6,8–12,27,28,37–40). The rodent models include those without immune defects and those in which immunodeficiency is induced with drug or radiation therapy, as well as rodent species lines with specific defects in both humoral and T-cell immunity.
4. During the period of neutropenia animals are housed in filter top cages. We have not found concomitant antibiotic therapy necessary to prevent bacterial superinfection over the period of study ranging from 24 to 96 h. In addition, we do not provide sterile bedding, food, or water to the animals.

5. In treatment of infections the concentrations of drug at the site of infection are most important. Numerous investigations have detailed tissue homogenate kinetics with a variety of antifungals. However, treatment outcomes at all noncentral nervous system infection sites have correlated equally well with serum pharmacokinetics as with tissue pharmacokinetics. The majority of *Candida* organisms at infection sites are extracellular or within the interstitial space. Serum concentrations have been shown to be an excellent surrogate of interstitial drug concentrations. Thus, it is not surprising that these measurements have correlated well with treatment efficacy. Furthermore, tissue homogenate kinetic measurements and interpretation are fraught with a variety of difficulties including variable tissue binding among drugs and the mixing of both intracellular and extracellular volumes which can overestimate or dilute the true extracellular tissue estimate depending on the degree of intracellular drug concentrations.

6. Microbiological assays rely on the antimicrobial activity of a drug in an agar-based system to inhibit the growth of an indicator organism (41). The diameter of inhibition zones is correlated with spiked standard concentration zones. The advantages of this system include low cost and the ability to measure active drug, including metabolites that may possess microbiological activity. One disadvantage of microbiological assays is a relatively high limit of detection. The level of detection can be higher than the MIC of the organisms for which the drug is intended. The other method type includes a variety of chromatography assays, most commonly HPLC. The advantage of this system is enhanced detection of low concentrations relative to the microbiological system and separation of drug and metabolites. However, a larger blood volume is usually required for an HPLC assay. This requires blood from an entire mouse per time point. Thus, much larger numbers of mice are required and the kinetic calculations are from groups of mice instead of in individual animals.

7. There are a variety of methods for measuring the extent of antifungal drug-protein binding (45). Most methods rely on separation of the protein bound drug from the unbound fraction by physical means. The methods available to accomplish this include ultrafiltration, equilibrium dialysis, ultracentrifugation, microbiological assays, and several more recently described commercial separation columns.

8. The timing of start of therapy can be varied depending on the questions being addressed relative both to the intent of antifungal drug therapy and the infection model (12). Starting therapy immediately after infection mimics preventative therapy. The longer the time between infection and antifungal therapy, the greater the amount of growth in animals and the more difficult therapy. A 2-h delay between infection and therapy allows the organism to enter the log phase of growth.
9. There are two factors considered in the choice of drug levels. Most importantly the doses should produce serum levels in the animals that one would anticipate in humans to allow one to observe in vivo activity at therapeutic levels. Second, doses much higher and lower than this are utilized to optimize the range in efficacy from no effect to maximal effect. This allows one to characterize the entire dose–response relationship in vivo. In our experience this can be accomplished with five or six drug levels that vary in dose two- to fourfold, such that the range in total dose levels will range from 10- to 24-fold.

10. The duration of therapy is dependent primarily on the PKs of the antifungal drug. In PD analysis of antimicrobials, dose fractionation studies allow one to determine the relative efficacy of different dosing frequencies. The minimal fractionation scheme would include a five total dose levels split into a single dose, two doses, and three doses. The shortest dosing interval most often is chosen to be greater than the elimination half-life to minimize the impact of drug accumulation. Thus the minimal duration of therapy will be at least three half-lives. The minimal duration we study is a 24-h treatment period.

11. Survival frequently is used as a treatment end point, either in place of or in addition to organism burden (21,23,24,27,29). Numerous investigations have demonstrated that treatment efficacy is similarly predicted by either quantitative culture or survival. The former requires fewer animals and most often a shorter period of study. In addition, many animal review committees now hesitate to allow animal survival as an end point. In place of actual animal survival, the investigator monitors the animals for signs of distress, at which point euthanasia is undertaken.

12. Other internal organs, including the liver, spleen, brain, and lung, have been utilized for endpoint CFU/tissue measurements (6,27,29,31,33). However, the burden of growth is much higher in kidneys, relative to other organs and continues to increase during a 72-h study period in the absence of antifungal therapy. During a 24- to 72-h period, the burden in kidneys can reach 10^7–8 CFU/kidneys. Another convention is to express organism burden as CFU/g of tissue (10,23,24,29,31). There can be variation in burden between the left and right kidney, however, there is not significant variation from animal to animal when burden is considered as CFU/2 kidneys per animal. This eliminates the need to weigh each kidney individually and calculate burden based on weight (45).

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References


Murine Model of Invasive Aspergillosis

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Summary

The growing importance of infectious caused by Aspergillus species during the last decade has created a need for practical and reproducible animal models of invasive aspergillosis suitable for studying fungal virulence, infection pathogenesis, diagnostic markers, and testing of antifungal therapy. Murine models remain the most commonly used models for studying aspergillosis because of their ease of manipulation and the large number of reagents available for studying disease–host responses. This chapter provides describes a murine model of invasive aspergillosis suitable for basic and translational studies of invasive pulmonary aspergillosis and highlights experimental variables that affect the course and reproducibility of infection.

Key Words: Mouse; Aspergillus; murine; animal model; aspergillosis.

1. Introduction

During the last 20 yr, the opportunistic pathogen Aspergillus fumigatus has emerged from a saprophytic fungus of minor medical interest to one of the most common causes of infectious disease-related death in immunocompromised patients (1,2). The spectrum of syndromes related to Aspergillus range from allergic bronchopulmonary disease associated with airway colonization to invasive infection that can spread to virtually any organ in the body. The most common life-threatening manifestation of invasive aspergillosis in humans, however, is a hemorrhagic pneumonia that develops in patients after persistent neutropenia and/or prolonged iatrogenic suppression of cellular immunity with adrenal corticosteroids (1). Depending on the depth and duration of immunosuppression, attributable mortality rates for invasive aspergillosis range from 60 to 90% (1,2).

The growing importance of infectious caused by Aspergillus species during the last decade has created a need for practical and reproducible animal models of invasive aspergillosis suitable for studying fungal virulence, infection patho-
genesis, diagnostic markers, and testing of antifungal therapy. Invasive aspergillosis has been established in fruit flies, mice, rats, rabbits, guinea pigs, chickens, cows, turkeys, ducks, and monkeys (3). However, mice remain the most common animal species for the study of invasive aspergillosis for several reasons:

1. Mice are the most phylogenetically reduced species useful for the study of antifungal efficacy and toxicity.
2. Mice are relatively inexpensive and easy to house/manipulate.
3. A large number of reagents, including recombinant proteins and cytokines, are available for the mouse species.
4. Mouse species with natural mutations in innate and adaptive immunity are widely available and thus are ideal model systems for forward screening of genes that have a role in the susceptibility or resistance to infection.
5. Transgenic or targeted gene knockouts in mice have been successfully used to characterize the mechanisms of gene action during infectious processes.

Despite the frequent use of mice as an animal model system for studying invasive pulmonary aspergillosis (IPA), no consensus exists with respect to the best experimental methodology to induce infection in these animals (3). Considerable variation can be found in the medical literature with regards to animal selection (age, weight, sex, and strain), underlying immunosuppression, and inocula (inoculum concentration, route of administration). In an extensive review on invasive aspergillosis, Latge summarized the following key factors in murine models of invasive aspergillosis (3):

1. Irrespective of genetic background, most mouse strains are equally susceptible to developing infection with *A. fumigatus*. However, some differences in the rapidity of disease progression and response to antifungal therapy can be seen between outbred Swiss mice and inbred strains (e.g., BALB/c, C57BL/6, CDF21). For example, we have observed that BALB/c mice, unlike outbred Swiss mice, require no corticosteroid treatment to establish a uniformly lethal infection. Animal weight can also play a role in infection, as heavier animals often require a higher inoculum to establish infection. Ideally, animals should weigh approx 20–30 g at the time of infection.
2. Intranasal (IN) or intratracheal (IT) inoculation generally is the preferred route of infection because this method mimics the natural sinopulmonary route of infection in humans (3). However, immunocompetent mice are relatively resistant to infection and can clear as many as 10^8 conidia without developing disease (4). For this reason, immunosuppression typically is needed to establish IPA in animals. Outbred Swiss mice often require either higher inocula or corticosteroid pretreatment to reduce the coinciding activity of pulmonary macrophages (3,4).
3. As with other animal infection models, the inoculum or dosage of conidia is the single most important factor (in immunosuppressed animals) that defines the
lethality and rapidity of infection (3). Changes in the severity or lethality of infection are often best controlled through changes in the inoculum.

4. As with humans, immunosuppression substantially increases the susceptibility of mice to infection. Cyclophosphamide and cortisone acetate (alone or in combination) are the most common immunosuppressive regimens used in mice. Repeated dosages of cyclophosphamide require that the animals be housed in a sterile environment and receive supplemental antibiotics to reduce the risk of bacterial infections.

5. Outside of animal death, the most common end point of disease burden in murine models of IPA is tissue fungal burden as reflected by the colony-forming units (CFU) recovered from cultures of homogenized animal tissue. Because most Aspergillus’ do not produce spores during invasive disease and mycelia are difficult to count by direct serial culturing of infected organs, CFU counts may decrease as fungal disease burden increases (3,5). For this reason, quantification of fungal burden by CFU counts often does not represent a sensitive index of disease burden, particularly late in the course of active disease (5). Chitin, a major structural component of Aspergillus hyphae cell walls, can be extracted from harvested tissue, converted to glucosamine, and measured using a colorimetric assay (6). Although this assay is extremely labor intensive, it has been shown to accurately reflect Aspergillus growth in vivo and is a sensitive index of imminent animal death (6). More recently, quantification of Aspergillus-specific nucleic acids using real-time polymerase chain reaction (PCR), has been shown to be a sensitive index of Aspergillus disease burden over 6-logs of magnitude and probably represents the most sensitive method for quantifying disease burden in organs of animals with IPA (5).

2. Pathogenesis of Disease in a Murine Model of IPA

Because Aspergillus is a saprophytic mould that colonizes the respiratory tract of an immunocompromised host, the pathogenesis of invasive infection can be defined by the underlying host immunosuppression (Fig. 1 [7]). Pulmonary alveolar macrophages ingest and kill inhaled conidia, whereas polymorphonuclear (PMN) leukocytes and monocytes are fungicidal to the hyphal form of Aspergillus (8,9). If either line of these innate host cellular defenses are impaired by corticosteroids (macrophages, neutrophils) or cyclophosphamide (neutropenia), A. fumigatus can germinate into hyphae and invade the lung parenchyma and surrounding blood vessels (9).

Once A. fumigatus has invaded into lung tissue, development of an appropriate T-cell response is critical for control of the infection. Animals that develop a strong T-helper cell Type 1 (Th1) response characterized by increased production of inflammatory cytokines interferon (IFN)-γ, interleukin (IL)-2, and IL-12 and stimulation of cellular immunity, will demonstrate greater resistance to invasive infection and healing patterns of disease (10,11).
Alternatively, animals that develop pronounced Th2 responses, which are characterized by decreased production of IFN-γ and increased production of IL-4, IL-10 and stimulation of humoral responses, will exhibit progressive infection and nonhealing patterns of disease (10,11). Subtle differences in Th1/Th2 responses may play a role in susceptibility to IPA among different mouse species. We have noted that outbred Swiss mice are much more likely to mount strong Th1 responses after sinopulmonary challenge with Aspergillus conidia compared with inbred, Th2-leaning species, such as BALB/c mice. This difference may explain why corticosteroid pretreatment, which blunts the Th1 response, is necessary to establish IPA in outbred Swiss mice.

On histopathology, acute IPA appears as an aggregate of mycelium surrounded by radiating hyphae or sheets of compact hyphae growing in one direction are evident in congested or hemorrhaging lung tissue (Fig. 2 [12]). The septate hyphae of aspergilli have dichotomous branches at acute (45°) angles and are of uniform width (3–6 µM). If mycelia have invaded blood vessels, infection may disseminate to other organs, including the brain, kidneys, liver, and spleen. However, the most common cause of death in mice is progressive respiratory failure associated with damage in the lung. In neutropenic
animals, pronounced respiratory difficulty may not be observable until 24 h before death. Therefore, animals require frequent monitoring once respiratory difficulty begins and should be aggressively euthanized to minimize animal discomfort. Occasionally, mice may present initially with central nervous system manifestations 12–24 h before death, which is indicative of disseminated IPA (rolling, circling, paresis of hind legs), which should prompt immediate euthanization of the animal.

3. Materials
   1. Cyclophosphamide (Sigma cat. no. CO768).
   2. Tween-20 (Sigma cat. no. P7949).
   3. Tetracycline (Sigma cat. no. T 7660).
   4. Potato dextrose agar (PDA) or Sabouraud dextrose (SD) agar plates or slants.
   5. High-speed centrifuge.
   6. Hemocytometer slide.
   7. Microscope.
   8. 1-mL Syringes and 27 1/2-gage needles.
9. 10-mL Syringes.
10. Scalpel, surgical scissors, and clamps.
11. 20–100 µL Micropipetor.
13. Bead-Beater homogenizer (e.g., Mini-Beadbeater, Biospec, Bartlesville, OK).
14. Sterile 1- to 2.5-mm diameter glass beads (Biospec).
15. Sterile screw-top tubes (15 mL).
16. Isoflurane/oxygen nebulizer or other approved anesthetic method.
17. Sterile glass wool.
18. Phosphate-buffered saline (PBS).
19. Deoxyribonucleic acid (DNA) extraction/purification kit (e.g., DNeasy tissue kit, Qiagen, Valencia, CA).

4. Methods

The following methods describe inoculum preparation, animal immunosuppression, animal infection procedures, tissue harvest, and methods for quantifying *Aspergillus* fungal burden in lung tissue.

4.1. Inoculum Preparation

Because the inoculum is a critical variable that defines the lethality and rapidity of infection in the animals, special attention should be directed towards the correct preparation of a conidial suspension.

1. A stock conidial suspension of *Aspergillus* should be swabbed on three to five PDA plates or 6–10 agar slants 1 wk before inoculation.
2. Plates or slants are incubated at 37°C in a humid chamber for 3–5 d or until mature growth (thick fungal lawn) is evident.
3. Inoculum preparation should begin at least 1 d before infection.
4. Conidia are harvested by flooding plates with 5 mL of PBS solution containing 0.2% Tween-20. The surface of the mycelial mat is rubbed gently with a sterile loop or sterile cotton swab, and the resultant suspension is transferred with a sterile pipet to a 10-mL syringe that is loosely packed with sterile glass wool.
5. The harvested suspension is then filtered through the glass wool to remove hyphal fragments and the elution (conidia suspension) is collected into a separate tube. Typically, the concentration of the elution is approx 1 × 10^6 conidia/mL.
6. Depending on the number of animals to be inoculated, this procedure may need to be repeated several times to collect sufficient numbers of conidia.
7. The conidia suspension are then washed in sterile PBS + 0.02% Tween-20 by centrifugation and adjusted to a final conidia density of 5 × 10^6 CFU/mL using a hemocytometer.
8. The final viable inoculum of spores is confirmed by plating serial dilutions of the conidia suspension on PDA. The prepared inoculum can be stored at 4°C for 24 h before to inoculation of the animals.
4.2. Animal Immunosuppression

1. Pathogen-free female BALB/C mice (20–28 g) housed in filter top cages with sterile bedding, food, and water are used to establish infection. Animals are allowed access to food and water ad libitum throughout the experiment and should be weighed daily during experiments.

3. Prior to cyclophosphamide treatment, tetracycline (1 mg/mL) is added to the water to reduce the risk of bacterial infections during immunosuppression and is changed daily.

3. Cyclophosphamide solution is prepared in sterile saline to a concentration (15 mg/mL) that allows simple calculation of doses based on animal weight (e.g., for an animal weighing 25 g; 0.025 kg [mouse weight] \( \times \) 150 mg·kg\(^{-1}\) [cyclophosphamide dose] = 3.75 mg/15 mg/mL [cyclophosphamide solution] = 0.25-mL dose). Cyclophosphamide is injected intraperitoneally using a 27 1/2-gage needle on days –4 and –1 before inoculation, and then every three days thereafter (Days +3, +6, +9) to maintain neutropenia. Mice will typically be neutropenic (<0.5 \( \times \) 10\(^{-9}\) cells liter\(^{-1}\)) 3 d after the initial injection of cyclophosphamide (13). Without subsequent injection, animals will recover from neutropenia in 3–4 d.

4.3. Animal Inoculation

1. Animal inoculation should be performed in a class II biosafety cabinet approved for flammable anesthetics.

2. On day 0, neutropenic mice are anesthetized by the inhalation of 5% isoflurane:oxygen in a nebulizer chamber. If isoflurane or a nebulizer chamber is not available, other anesthetics administered by inhalation (e.g., diethyl ether) or injection (e.g., ketamine hydrochloride/xylacine) can be substituted (13). Anesthesia needs to be of sufficient depth to render the animal unconscious for \( \geq 1 \) min and suppress the swallowing reflex. However, if anesthesia is too deep, the animal may develop cardiac arrest owing to the vagal response that occurs when the inoculum is dosed into the trachea (13).

3. Once the animal is anesthetized, the mouse is held upright and a 30 µL inoculum is delivered by means of a pipet into one nostril to allow contralateral breathing during inoculation (Fig. 3). The pipet tip is held just above (1–2 mm) the nostril to produce fine droplets (0.5–1 mm diameter) that can easily enter the nose and do not choke major airways. Hyperventilation after inoculation is common in mice and is a welcome side effect, as it results in greater distribution of the inoculum throughout the lungs (13). After three to five breaths, crackles during deep inspiration may be felt against the hand as the inoculum is pulled into the lungs. Mice should be held upright for an additional minute or until consciousness is regained, whichever comes first. The animal is then placed in the cage with the head and thorax slightly elevated until awake.

4. Using this procedure, approx 75–85% of the inoculum will be delivered to the lung (13).
4.4. Animal Monitoring/Course of Infection

1. Depending on the inoculum delivered and the isolate used for infection, animals will begin to manifest signs of infection 48–72 h after inoculation. Early nonspecific signs of infection include inactivity, rough/matted fur, conjunctivitis, or diarrhea. Respiratory difficulty and use of accessory muscles during respiration will become more evident as infection progresses. Animals with impaired ambulation, evidence of emaciation or muscle atrophy (outside of body weight), skin lesions (e.g., ulcerative dermatitis), or any obvious illness (severe lethargy, anorexia, bleeding, severe breathing difficulty), or evidence of central nervous system involvement (rolling, paresis, circling) should be immediately euthanized and death can be recorded as occurring 12 h later.

2. Daily animal weights should decrease in animals with progressing infection; however, some weight loss is not uncommon in uninfected animals receiving cyclophosphamide (13).
3. Animals are euthanized by carbon dioxide narcosis.
4. Typically, 90% of animals will succumb to infection by 10 d.

4.4.1. Study End Points

1. Major parameters of the model are lethality and time to death. Lung fungal burden can be a complimentary or primary end point, depending on study objectives.
2. Terminal samples or organs can be harvested at the time of euthanization (or immediately after) depending on the objectives of the study.
3. Blood (0.6–1 mL) typically is harvested via the cardiac puncture procedure during euthanasia procedures (100% CO₂ narcosis for 2 min) or under anesthesia before euthanasia.
4. Bronchial alveolar lavage can be performed postmortem. A detailed explanation of procedures for carrying out cardiac puncture or bronchial alveolar lavage is beyond the scope of this chapter but can be found in other sources (available at: http://research.uiowa.edu/animal/ [13]).
5. Histopathology should be performed routinely in a subset of animals to confirm the presence of invasive aspergillosis and corroborate other study end points. Special care must be taken when harvesting lungs for histopathology. Prior to removal from the animal, a 23-gage needle is inserted into the trachea and 1 mL of fixative (e.g., 10% neutral formaldehyde) is slowly infused to inflate the lung. The inflated lung is then carefully removed, and placed in 5–10 mL of fixative for further processing and embedding.

4.5. Measurement of Lung Fungal Burden

Analysis of lung fungal burden is the most direct parameter for following the course of infection and can be a useful tool for characterizing sub-lethal infections. The principal disadvantage of using lung fungal burden as a primary end point, however, is that animals must be euthanized in order to obtain this information. As previously mentioned, methods used for bacteria and yeast enumeration in tissue (i.e., direct semiquantitative culturing or CFU determination) are not accurate methods for assessing fungal burden in aspergillosis, as CFU counts do not discern between invasive hyphal forms and colonizing (noninvasive) conidia as infection progresses (5). Serial plating of tissue homogenate on chloramphenicol containing-agar (to reduce bacterial colonization) for CFU determination may provide some indication of disease burden early in the course of infection.

Chitin is a major component of the fungal cell wall and can be assayed in mouse tissues. Lehman and White demonstrated that measurement of chitin in mice with invasive aspergillosis provides a sensitive index of disease burden and correlates with animal death (6). Briefly, homogenized tissue is treated with hot concentrated alkaline solution to form the insoluble product chitosan. An aldehyde product of chitosan is then made on reaction with NaNO₂, and the
aldehyde groups are assayed colorimetrically as glucosamine equivalents vs a glucosamine standard. The major drawbacks to chitin assay are the long sample preparation time (~2 d) and limited sensitivity of the assay to detect low fungal burden.

4.5.1. Measurement of Aspergillus Lung Fungal Burden by Quantitative PCR

More recently, measurement of *Aspergillus* nucleic acid in animal lung by quantitative PCR has been shown to be a sensitive indicator of lung fungal burden and disease progression (5). Unlike CFU counts, lung fungal burden measured by quantitative PCR steadily increases until a threshold of approx $1 \times 10^6$ to $1 \times 10^7$ conidia equivalent of *Aspergillus* DNA, which often coincides with animal death. The dynamic range of fungal burden detection is relatively large for quantitative PCR, and a standard curve can be constructed more than 6 logs of magnitude of *Aspergillus* DNA. For these reasons, this method has become the preferred approach in our laboratory for determination of *Aspergillus* fungal burden in tissue.

Quantitative PCR analysis of *Aspergillus* lung fungal burden is performed as follows:

1. Lungs are harvested from animals and stored in 2-mL cryogenic vials at –70°C until homogenization and lysis can be performed. Before homogenization, tissue is thawed and 1 mL of saline is added to each sample.
2. To correct for potential differences in percent DNA recovery, saline can be spiked with an appropriate internal standard for amplification by real-time PCR to correct for potential intersample differences in DNA recovery. For example, our laboratory uses saline containing $5 \times 10^8$ copies/mL of an internal standard plasmid to correct for differences in DNA recovery (Genbank-AF17244). When this sequence is amplified in separate reactions, the CT value of amplification should be similar for all curves, otherwise a correction factor must be applied to account for differences in DNA purification/recovery of the internal standard.
3. Sterile glass beads (1–2.5 mm) are then added to the cryovial containing tissue and saline. Infected tissue is then disrupted by vigorous agitation (three bursts at 4200–4800 rpm for 20–30 s) using a mini Bead-Beater homogenizer. Agitation of samples should be alternated with 10 s of cooling in an ice water bath to minimize sample heating. The homogenate supernatant (displaced by glass beads) is then transferred to separate vials and DNA can be isolated using a standard kit or methods (e.g., DNeasy tissue kit, Qiagen).
4. Briefly, 90 µL of tissue homogenate is lysed in a buffer containing proteinase K for 12 h at 55°C. Lysed samples are then applied to silica columns and DNA is purified and eluted with a series of buffer washes using a high-speed centrifuge. DNA recovered in 200 µL of elution buffer is then stored at –20 to –70°C until analysis. Unused homogenate can be stored at –70°C and reanalyzed if necessary.
5. Quantification of *Aspergillus*-specific DNA is performed by real-time PCR assay as described by Bowman and colleagues (5). This assay requires oligonucleotide
amplification primers and a dual-labeled fluorogenic oligonucleotide hybridization probe complementary to a sequence from the *Aspergillus* 18S rRNA gene (Genbank-AB008401). The dual-labeled fluorogenic probe consists of a reporter and quencher dye, which suppresses the reporter fluorescence when the probe is intact. However, during PCR, the probe is cleaved by the 5'-3' nucleolytic activity of the DNA polymerase, resulting in a fluorescent signal which is amplified and measured by detector in a real-time PCR thermocycler. Oligonucleotides sequences for the primers and probe are as follows:

Forward primer, 5' –GGCCCTTAAATAGCCCGGT-3'
Reverse primer, 5'–TGAGCCGATAGTCCCCCTAA-3'
Hybridization probe, 5'–FAM-AGCCAGCGGCCGCAAATG-TAMRA-3'

Each reaction consists of 5 µL of DNA template, 900 nM of forward and reverse primers, 200 nM of the hybridization probe, and TaqMan Universal PCR Master Mix (Applied Biosystems, Framingham, MA) containing Taq DNA polymerase and requisite buffers, and molecular grade water adjusted for a final volume of 25 µL. The thermocycler protocol consists of the following:

1. A holding stage of 2 min at 50°C.
2. A holding stage of 10 min at 95°C.
3. 40 reaction cycles of 15 s at 95°C alternating with 1 min at 60°C.

The reaction is monitored in real time using the ABI PRISM 7000 sequence detection system (Applied Biosystems) and Sequence Detection software (version 1.0; Applied Biosystems) that assigns a C_T value that identifies the cycle number during PCR when fluorescence exceeds a threshold value. These values are then used to calculate conidial equivalents (CE) from a six-point standard curve generated from DNA samples prepared by spiking naïve mouse lungs with known amounts of *Aspergillus* conidia (10^2–10^7 conidia; Fig. 4). DNA samples and the standard curve are assayed by quantitative PCR in duplicate.

5. Troubleshooting

The two most common problem investigators face when starting experiments with a murine model of invasive aspergillosis are the lack of an ability to establish a uniform infection through the sinopulmonary route and early animal death caused by rapidly progressing, hyperacute infection. If inoculum preparation and immunosuppression are performed correctly, then both problems can be corrected by adjusting the concentration of the inoculum. In animals that die rapidly owing to overwhelming aspergillosis, a 1–2 log reduction in the concentration of inoculum should extend mean survival time by 3–7 d. Lack of animal death or uniformity in infection should initially be addressed by escalation of the challenge inoculum in 1-log steps. Superconcentration of the original conidial suspension harvested from culture will be required if the target inoculum exceeds 1 × 10^7 CFU/mL (4). In some mouse strains (e.g.,
Fig. 4. Relationship of animal survival and fungal burden as determined by quantitative polymerase chain reaction (PCR). (A) represents the survival curve for control animals (infected but not treated with antifungals) and animals receiving antifungal therapy active against invasive aspergillosis (amphotericin B, 1 mg/kg intraperitoneal injection). Each group contains 10 mice. (B) represents a typical standard curve that is prepared by spiking naive lung tissue with known concentrations of *Aspergillus* conidia. Units are expressed in terms of conidia-equivalent *Aspergillus* DNA. (C) Comparison of fungal burden in control mice and mice treated with antifungal therapy. AMB, amphotericin B.
outbred Swiss Mice), even high inoculum concentrations (e.g., $1 \times 10^8 \text{ CFU/mL}$) may be insufficient to overcome pulmonary macrophage defenses. To enhance host susceptibility, cortisone acetate (Sigma, cat. no. C-3130) can be administered as a single subcutaneous 250 mg/kg dose 1 d before infection. When preparing the cortisone acetate injection in sterile saline, it is important to wet the weighed powder with several drops of Tween-20 to ensure adequate dispersion of the insoluble steroid in solution. The insoluble (but homogenous) suspension is then injected subcutaneously and acts as a “depo” steroid injection during the course of the experiment. Pretreatment with corticosteroids significantly reduces the challenge inoculum required to induce invasive pulmonary aspergillosis in mice.

If infection severity and uniformity are still suboptimal after inoculum escalation and corticosteroids, inoculation technique should be suspected. As mentioned previously, it is important to verify that the inoculum is inhaled rather than swallowed. A larger volume of inoculum (e.g., 50 µL) may improve delivery to the lungs in some mouse species.

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References


Antifungal Combinations

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Summary

The increase in fungal infections and the change in fungal epidemiology is caused by the extensive use of antifungal agents to treat fungal infections that are being diagnosed in severely immunocompromised hosts. In addition, opportunistic fungal infections resistant to antifungal drugs have become increasingly common, and the armamentarium for treatment remains limited. A possible approach to overcoming these problems is to combine antifungal drugs, especially if the mechanisms of action are different. The in vitro test is the first step to evaluate possible antifungal combinations. In this chapter, the three most frequently used methodologies are described: checkerboard, E-test, and time-kill curves. The description of each technique and interpretation of the results are addressed in detail.

Key Words: Yeasts; filamentous fungi; antifungal combinations; FICI; E-test; kill curves; Aspergillus; Candida.

1. Introduction

Antimicrobial combinations are used most frequently to provide broad-spectrum empiric coverage in the treatment of infections in patients who are seriously ill. Advantages of antimicrobial combination therapy include the possibility of decreasing the emergence of resistant strains, decreasing dose-related toxicity as a result of reduced dosage and, most importantly, achieving antimicrobial synergism. Less frequently, combination of antimicrobials are chosen because an identified pathogen is resistant to the conventional dose of single antimicrobial.

The mortality rate of invasive fungal infections is high despite antifungal chemotherapy, monotherapy with amphotericin B (AMB), azoles (fluconazole, itraconazole, or voriconazole), or caspofungin. Therefore, new approaches for treating these infections are warranted. Promising results are coming from the field of in vitro combination of antifungal drugs against fungi (1–4).
In this chapter, different methodologies to evaluate the in vitro antifungal interactions are described by the most frequent techniques, such as checkerboard, E-test, and the time-kill curves.

2. Materials

2.1. Checkerboard Technique

1. RPMI-3-\((N\text{-morpholino})\) propanesulfonic acid (MOPS) medium: dissolve 10.4 g of RPMI-1640 powder (with glutamine and phenol red, but without bicarbonate) in 900 mL of distilled water. Add 34.53 g of MOPS (0.165 mol/L final) and adjust the pH to 7.0 with NaOH. Make up to a final volume of 1 L with distilled water. Filter sterilize and store at 4°C.
2. Sabouraud’s dextrose (SD) and potato dextrose agar (PDA) slants.
4. McFarland scale 0.5.
5. Hemocytometer.
7. 96-Well, flat-bottom microtitration plates.
8. 25 cm² Polystyrene tissue culture flasks.
9. Antifungal drugs: AMB, fluconazole (FLU), itraconazole (ITZ), voriconazole (VRZ), 5-flucytosine (5FC), caspofungin (CAS), or any other investigational drugs. A powder of known potency should be used.

2.2. E-Test Method

1. RPMI-1640–MOPS medium (2X) dissolve 20.8 g of RPMI-1640 powder (with glutamine and phenol red but without bicarbonate) in 900 mL of distilled water. Add 69.06 g of MOPS and adjust the pH to 7.0 with NaOH. Make up to a final volume of 1 L with distilled water. Sterilize by filtration.
2. Spectrophotometer reader.
3. McFarland scale 0.5.
4. Antifungal drugs: E-test strips provided by the manufactured.
5. Sterilized Petri dishes that are 15 cm in diameter.
6. SD agar.
7. PDA agar.

2.3. Time-Kill Curves

1. RPMI-MOPS medium: dissolve 10.4 g of RPMI-1640 powder (with glutamine and phenol red, but without bicarbonate) in 900 mL of distilled water. Add 34.53 g of MOPS (0.165 mol/L final) and adjust the pH to 7.0 with NaOH. Make up to a final volume of 1 L with distilled water. Filter sterilize and store at 4°C.
2. SD slants.
3. Hemocytometer.
4. SD plates.
5. Sterile spreaders (Hockey sticks).
6. Orbital shaker.
7. 25 cm² Polystyrene tissue culture flasks.
8. Antifungal drugs: an antifungal powder of known potency should be used.

3. Methods

3.1. Checkerboard Technique

1. Prepare concentrated antifungal stock solutions and store in aliquots at −80°C. Most of the antifungal drugs can be stored at −80°C for up to 6 mo without loss of activity (see Note 1 [5,6]).

2. For two-dimensional microplate preparation, make a series of 2-fold dilutions of each drug in the corresponding solvents according to the dilution scheme of the National Committee for Clinical Laboratory Standards (NCCLS) and then dilute 100-fold in the medium to obtain four times the final concentration. Add 50-µL aliquots of each drug concentration of the drug A to columns 1 to 11, and then 50-µL aliquots of each concentration of drug B to rows A to H. In the wells of column 11 and in the wells of row H, add 50 µL of the medium containing 1% of the solvent. Thus, column 11 and row H contain the drugs A and B alone, respectively. Column 12 are the drug-free wells that serve as the growth control (Fig. 1).

3. Inoculum preparation for yeasts: culture each isolate for 1–2 d on SD agar at 35°C. Take some colonies and suspend in sterile water. After, vortex vigorously, and adjust the turbidity to 75–77% of transmittance at 530 nm or adjust the yeast suspension to 0.5 McFarland scale. Then dilute the suspension 1/1000 in RPMI-1640 medium, which results in a 2X inoculum. Therefore, the final inoculum will range from $0.5 \times 10^2$ to $5 \times 10^3$ colony-forming units (CFU)/mL.

4. Inoculum preparation for molds: culture each isolate for 7 d on PDA medium for 5–7 d at 35°C. Collect conidia or spores with a swab and suspend in sterile saline plus Tween-20 (0.05%). After the heavy particles are allowed to settle, measure the turbidity of the supernatants spectrophotometrically at 530 nm and adjust the transmittance to 80–82% for Aspergillus and Sporothrix, 68–72% for Scedosporium and Fusarium species. In the case of Zygomycetes or other uncommon fungi, count spore suspensions with a hematocytometer and then dilute into RPMI-1640 to a concentration of $2 \times 10^4$ spores/mL (see Note 2). Dilute each suspension 1:50 in RPMI-1640 to obtain two times the final inoculum size, ranging from $0.5 \times 10^4$ to $5 \times 10^4$ CFU/mL. Verify the inoculum size by plating 100 µL of serial dilutions of each inoculum onto a SD agar plate and incubate until growth became visible.

5. Inoculate each microplate with 100 µL of the inoculum suspension.
6. Incubate the microplates at 35°C for 48–72 h (see Note 3 [5–7]).
7. Minimum inhibitory concentration (MIC) end point determination: can be determined visually and/or spectrophotometrically at 450 nm for yeasts and 405 nm for molds. In general for spectrophotometric reading the optical density (OD) of
synergism

additivity

antagonism

Fig. 1. Checkerboard technique: interpretation of the results.
the blank, a microtitration plate that is incubated with a conidium-free inoculum, is subtracted from the OD values. The percentage of growth for each well is calculated by comparing the OD of the well with that of the drug-free control. The MIC for azoles and 5FC is defined as the lowest concentration showing prominent growth inhibition (MIC–2: 50% reduction) against *Candida* and *Cryp-
tococcus*. The MIC for ITZ, voriconazole, posaconazole, and AMB against molds are defined as the lowest concentration showing no growth (MIC–0: 100% reduction) according to NCCLS guidelines (5,6). The MIC end point for CAS is not yet standardized. Both on-scale and off-scale MICs should be included in the analysis. High off-scale MICs should be converted to the next twofold dilution, whereas low off-scale MICs are left unchanged.

8. To evaluate the interaction of the drugs, the fractional inhibitory concentration index (FICI) has to be calculated for each concentration. The FICI is calculated for each agent by dividing the inhibitory concentration of each drug when used in combination by its MIC. FICI values are calculated as follows: (MIC of Drug A in combination/MIC of Drug A alone) + (MIC of Drug B in combination/MIC of Drug B alone). The interpretation of the FICI is determined as follows: ≤0.5, synergistic effect; >0.5 but < 4, no interaction; and ≥ 4, antagonistic effect (see Note 4[8]). Among all FICIs calculated for each data set, the FICI is determined as the FICI\(_{\text{min}}\) (the lowest FICI) when the FICI\(_{\text{max}}\) (the highest FICI) is smaller than four; otherwise, the FICI is determined as the FICI\(_{\text{max}}\) (see Fig. 1).

### 3.2. E-Test Method

1. RPMI-1640–MOPS medium: dissolve 20.8 g of RPMI-1640 powder (2X) (with glutamine and 0.165 mol/L final solution) and adjust the pH to 7.0 with NaOH. Make up to a final volume of 1 L. Dissolve by heating 30 g of agar in 1 L of distilled water autoclave.

2. Heat the RPMI-1640–MOPS until 45°C and let the agar cool until 45°C. Then, mix both preparations in order to obtained 1X final solution and dispense in Petri dishes (25 mL for a 90-mm Petri dish; see Note 5).

3. Inoculum preparation for yeasts: to adjust the suspension, see Subheading 3.1., step 3. Dip a sterile swab into the yeast suspension and press out excess fluid. Inoculate the plates by swabbing the entire surface of the agar in three directions to achieve uniform distribution. Allow the moisture to be absorbed (15 min).

4. Inoculum preparation for molds: to adjust the suspension, see Subheading 3.1., step 4. Inoculate the plates in the same way as for yeasts. For a uniform distribution, after 1–2 min, aspirate the excess moisture and let the plate dry.

5. Place the antifungal strips onto the inoculated agar using sterile forceps.

6. Place the E-test strip (Drug A) onto the agar and remove it after 1 h (see Notes 6 and 7). Then place the E-test strip (Drug B) onto the agar over demarcation left from previous strip. Apply additional E-test strips for Drug A and B alone (Fig. 2).

7. Incubate the plates at 35°C for 48–72 h. The incubation time for *Candida* is 24–48 h; 48 h for *Aspergillus* and 72 h for *Cryptococcus neoformans*.
8. E-test susceptibility end points: MICs for azoles and 5FC are read at the intersection of the scale of the strip and the first discernible growth-inhibition ellipse according to the recommendations of the manufacturer. The E-test susceptibility end point MIC for AMB is reading at the intersection of the scale of the strip with the first completely clear ellipse.

9. For interpretation of the results and FICI calculation, see checkerboard methods in Subheading 3.1., step 8.

3.3. Time-Kill Methods

1. See Note 8.

2. Prepare concentrated antifungal stock solutions and store in aliquots at –80°C. Most of the antifungal drugs can be stored at –80°C for up to 6 mo without a loss of activity (see Note 9).

3. Culture the strain to be tested on SD agar slant for 24 h at 35°C.

4. Pick up a few colonies and make a suspension in 20 mL of sterile RPMI-1640–MOPS.

5. Count the yeast suspension in a hemocytometer.
6. Adjust the yeast suspension to $1 \times 10^6$ CFU/mL by adding the appropriate volume of RPMI-1640–MOPS.

7. Fill two or more tissue culture flasks with 17.8 mL of RPMI (for testing drugs alone) and two or more flasks with 17.6 mL of RPMI (for testing drugs in combination).

8. From stock solutions, prepare antifungal solutions at 100× the final desired concentration in the tests (see Note 10).

9. Add 200 µL of the 100X antifungal solution to obtain a 1X final concentration (see Note 11). At least four flasks must be used: two for the drugs alone, one for the drugs in combination, and one without any drug (control).

10. Inoculate each tissue culture flask with 2 mL of the yeast suspension at $1 \times 10^6$ CFU/mL to obtain a final inoculum size of $1 \times 10^5$ CFU/mL.

11. Incubate the flasks at 35°C with shaking for 48 h (see Note 12).

12. At predetermined time points (0, 1, 2, 4, 8, 24, and 48 h) aseptically remove 50-µL samples from each test solution, and dilute 1/10 and 1/100 in sterile NaCl 0.85% (see Notes 13 and 14). Plate 50 µL of each dilution (including the neat dilution) with a hockey stick on SD agar plates in duplicate. Incubate the plates at 35°C for 48 h.

13. Count CFU on each plate and calculate the mean CFU/mL (see Note 15).

14. Transform the CFU data to log_{10} and plot the mean log_{10}CFU/mL vs time (see Fig. 3).

15. Analyze the results by comparing the activity of the drugs alone with that of the drugs in combination as follows: an increase in killing of $\geq 2 \log_{10}$CFU/mL for the combination compared to the most active drug alone defined synergy. Antagonism is defined as $\leq 2 \log_{10}$CFU/mL decrease in killing with the combination, as compared with the most active single drug alone. Indifference is defined when the killing effect with the two agents in combination is within 2 $\log_{10}$CFU/mL of either agent alone (see Fig. 3 and Note 16).

4. Notes

1. Some antifungal agents can be dissolved in water (e.g., FLU, 5FC, CAS) but some others in dimethylsulfoxide (DMSO) (e.g., AMB, ITZ, voriconazole, posaconazole, and ravuconazole [5,6]).

2. For preparation of the inocula for Aspergillus species, Zygomycetes, and Scedosporium species, using a hemocytometer could be a better approach.

3. Generally, an incubation time of 24–48 h is used for Candida spp., 48 h for Aspergillus, 24 h for Zygomycetes, and up to 72 h for C. neoformans and Scedosporium species (5–7).

4. In practice, synergism or antagonism calculated as was pointed above is equivalent to a reduction or increase of at least two dilution steps in the MICs of both drugs when they are combined compared to the MICs for the drugs alone. However, there are no NCCLS guidelines for performing synergy studies and no standardization for analysis of the results. Then, criteria used to define synergism, additivity (or indifference), and antagonism may vary from one publication to another. According to the Loewe theory of drug interaction, in order to calculate
Fig. 3. Time-kill curves for assessment of in vitro antifungal combinations: interpretation of the results. An antifungal A with no activity against the tested strain is combined with antifungal B, C, or D. By comparison with the activity of the drugs alone, synergism, indifference, or antagonism is concluded for the combination. CFU, colony-forming units.
Antifungal Combinations

the FICI, the end point determination (MIC) for the drugs alone or in combination have to be the same; this is specially important when drugs with different end points, such as FLU and AMB against *Candida*, are combined.

5. To preserve the L-glutamine activity, is not possible to sterilize by autoclave the RPMI-1640 media. For that reason, to solve this problem, prepare the media and the agar at twice the final concentration and mix afterward. To facilitate the growth of the yeasts, addition of glucose, 2% final concentration onto the RPMI media is recommended.

6. According to the manufacturer’s instructions, 1 h is enough to achieve an effective release of the drugs from the carrier (E-test strip) into the agar matrix.

7. The E-test diffusion technique also can be set up differently when assessing in vitro antifungal combinations. For example, an E-test strip containing an antifungal A is placed on the agar plate containing an antifungal B at a concentration below the MIC against the tested strain. If the ellipse of inhibition decreases (corresponding to an increase of the MIC) antagonism is concluded and if the ellipse of inhibition increases (corresponding to an decrease of the MIC), synergy can be concluded.

8. It has to be noted that most of the fungi for which antifungal combination is of interest are classified as BSL (BioSafety Level)-2 organisms (e.g., *C. albicans* and several other *Candida* spp., yeast form of *C. neoformans*). Therefore, it is recommended that one manipulate the cultures and inoculated media in a safety cabinet.

9. Some antifungal agents can be dissolved in water (e.g., FLU, 5FC, CAS) and some must be dissolved in DMSO (e.g.. AMB, ITZ, voriconazole [6]).

10. With nonwater-soluble drugs that are dissolved in DMSO, the final concentration of solvent in the test will be 1%. This concentration of DMSO is assumed to have no activity against most of the fungi.

11. The final desired concentration in the test is variable depending on the susceptibility of the strains, the achievable drug level in serum in humans, and the chemical nature of the antifungal (fungistatic or fungicidal). Generally tests are carried out with final concentrations ranging between 0.25–16X MIC.

12. Some antifungal drugs are light-sensitive (e.g., AMB) and the flasks must be then incubated in the dark to avoid drug degradation during the incubation period. Incubation time depends on the species that are tested. Generally, an incubation time of 24–48 h is used for *Candida* spp. but can be extended to 72 h for *C. neoformans* (7).

13. For the controls and for the flasks containing antifungal drugs with no activity or tested at low concentrations a more important dilution factor must be used (e.g., 1/10^3 to 1/10^5) before plating.

14. For some antifungals used at high concentration (compared with the MIC of the tested strain) a drug carryover effect may be observed. Preliminary experiments should be performed to assess the presence of a carryover effect, which is defined as more than 25% reduction in CFU/mL in presence of the drug compared with the control value. Different techniques can be used to minimize this effect. For
example it has been shown that dilution followed by filtration of the samples is effective in eliminating the antifungal carryover (9).

15. The limit of quantitation of the technique also must be determined. In theory, this limit is 20 CFU/mL when undiluted samples of 50 µL are plated. It could be useful to determined experimentally the lower limit of accurately detectable CFU/mL (9).

16. At the present time, there are no NCCLS guidelines for performing synergy studies and no standardization for analysis of the results. Then, criteria used to define synergism, additivity (or indifference), and antagonism may vary from one publication to another.

References


Postantifungal Effect Methods

Roxana G. Vitale

Summary
Postantifungal effect (PAFE) is the evaluation of antifungal activity after the suppression of fungal growth when the drug is removed from the fungal suspension. In vitro, this effect might simulate the in vivo situation when the concentration of the drug falls to less than the minimum inhibitory concentration values and could be another tool, together with the classic in vitro susceptibility tests, to optimize the interaction of drugs–fungi. In this chapter, two model methods to evaluate the PAFE of yeasts and filamentous fungi are described in which practical advices and tricks are given to help the worker to develop the techniques. The procedures outlined include preparation of stock solutions of the drugs, concentration medium, exposure time colony count determination, and interpretation of the results to quantify the PAFE.

Key Words: Yeasts; filamentous fungi; postantifungal effect; PAFE; Aspergillus; Candida.

1. Introduction
Several in vitro methods to evaluate the action of different antifungal drugs against filamentous fungi and yeasts currently are used in which constant levels of the drugs are analyzed. However, in vivo, the organism is exposed to fluctuant levels of antimicrobial agents. In vivo, drug concentrations decrease to levels less than the minimum inhibitory concentration (MIC). In vitro, this situation can be simulated by removing the drug from the fungal suspension. The evaluation of whether any effect of the drug persists is called “post-drug exposure effect.”

The persistent suppression of micro-organism growth after the removal of antimicrobials was first studied for bacteria. The effect was called postantibiotic effect (1).

In this chapter, two model methods to evaluate the postdrug exposure effect for yeasts and molds, named the postantifungal effect (PAFE), are described in refs. 1–3, based on methods previously described for bacteria, yeasts, and molds.
Practical advices and tricks are written to help the worker develop the techniques.

The idea to evaluating whether certain drug display postantifungal effects against certain fungi is to have another tool together with the classic in vitro susceptibility tests to optimize the drug–fungi interaction. The in vitro evaluation with animal studies might provide a better understanding to correlate the in vitro tests with the in vivo outcome.

The method for yeasts differs in some steps from the method for molds because of their nonhomogeneous growth. The quantification of PAFE also is described differently for both groups.

The procedures outlined in this chapter are mostly confined to the preparation of stock solution of the drugs, the appropriate dilution in the medium to obtain the desired concentration, exposure time, washing steps for removing of the drug, monitoring of growth, colony count determination, and interpretation of the results to quantify the PAFE.

2. Materials

1. Fresh isolates of yeasts or filamentous fungi.
2. Antifungal powders: amphotericin B (AMB), which is sensitive to light, should be stored at 5°C. However, drugs such as itraconazole, fluconazole, and terbinafine can be stored at room temperature. For the storage of other drugs, follow the manufacturer’s instructions.
3. Solvents: dimethylsulfoxide (DMSO), water, or others according to the manufacturer’s instructions.
4. Stock solutions of the antifungal agents can be stored at –70°C until the day of use.
5. Medium: standard medium, RPMI-1640 with glutamine, without bicarbonate.
6. Buffer: 0.165 M of 3-(N-morpholino) propanesulfonic acid (MOPS).
7. Sterile saline solution.
8. Tween-20 or -80.
9. 20–1000-µL; 5, 10, and 25 mL pipet.
10. 100-µL Multichannel pipet.
11. 10-mL Plastic tubes adequate for centrifugation.
13. Petri dishes.
15. Hemocytometer chambers.
16. 96-Well, flat-bottom microtitration plates.
17. Computerized spectrophotometric reader.
18. McFarland 0.5 scale.
3. Methods

The methods described in this chapter explain how to evaluate the PAFE in yeasts and filamentous fungi. The quantification of PAFE is addressed in each case.

3.1. PAFE in Yeasts

1. Isolates. Pass the isolates by subculturing onto Sabouraud dextrose (SD) for 48 h at 37°C.
2. Determine the MIC according to the National Committee for Laboratory Clinical Standards (NCCLS [4]).
3. Prepare the stock solution of the antifungal agent at convenient starting concentration and storage at –70°C until use (see Note 1).
4. Dilute the stock solution at least 50 times in RPMI-1640 for nonwater-soluble drugs (see Note 2).
5. Prepare serial dilution from the above resulting starting drug concentration to obtain the desired concentration of the drug according to the MIC values for PAFE evaluation (see Note 3).
6. To prepare the yeasts for suspension, transfer some colonies from the 48-h culture in sterile water and adjust to 0.5 McFarland.
7. One milliliter of this suspension should be added to 9 mL of RPMI-1640 containing the desired concentration of the drug. Use plastic tubes adequate for centrifugation.
8. Prepare controls following the same steps without drug.
9. Incubate both, controls and the exposed yeasts at 37°C during the period of time that had been chosen.
10. After the incubation period, remove the drug by three sequential washings in sterile normal saline. Centrifuge for 10 min at 3500g each (see Note 4).
11. After the third washing, resuspend the pellet in sterile normal saline and readjust to 0.5 McFarland.
12. Add 1 mL of the readjusted fungal suspension to 9 mL of warm RPMI-1640.
13. Conduct colony count determination at predetermined time points. For instance, remove and aliquot from the controls and the exposed yeasts at 0, 6, 8, 10, 12, 14, 16, 18, 20, 24, and 26 h, and make 10-fold serial dilutions at each time for each condition. Streak 30 µL onto SD for 24 h (see Note 5).

3.2. PAFE in Filamentous Fungi

1. Isolates. Pass the isolates by subculturing onto SD or potato dextrose agar for 5–7 d at 28°C.
2. Determine the MIC according to the NCCLS (5).
3. Prepare the stock solution of the antifungal agent at convenient starting concentration and store at –70°C until use (see Note 1).
4. Dilute the stock solution at least 50 times in RPMI-1640 plus 0.5% Tween-20 for nonwater-soluble drugs (see Note 2).
5. Prepare serial dilution from the above resulting starting drug concentration to obtain the desire concentration of the drug that is according with the MIC values for PAFE evaluation (see Note 3).

6. Prepare the fungal suspension in a saline solution with 0.5% Tween-20 (see Note 6). After the particles are allowed to settle, transfer the supernatant to another tube, vortex, and make 10- and 100-fold dilutions. Establish the concentration of conidia or spores microscopically by hemocytometer chambers. Adjust the concentration to obtain $4 \times 10^5$–$5 \times 10^5$ conidia or spores/mL.

7. Add 1 mL from the adjusted suspension to 9 mL of RPMI-1640 plus 0.5% of Tween-20 with the concentration of the drug that had been chosen, such, 4, 2, or 1x the corresponding MIC value. The final volume should be 10 mL. Use plastic tubes adequate for centrifugation (see Note 7).

8. Prepare controls with 1 mL of fungal suspension and 9 mL of RPMI-1640 plus Tween-20, without drug.

9. Incubate both, controls and exposed conidia or spores at 37°C with shaking during a period of time that depends on the drug and the strain tested (see Note 9).

10. After the incubation period, wash the conidia or spores with saline plus Tween-20, centrifuge at 3500g for 15 min. Repeat for three wash cycles (see Note 4).

11. Decant the 98% of supernatant and resuspended the pellet with RPMI-1640 plus 0.05% Tween-20 to a final volume of 10 mL.

12. Make a colony count determination (colony-forming unit [CFU]/milliliter) of the control and the exposed conidia or spores by plating 30 µL of 10- and 100-fold dilution in water or saline onto SD agar plates. Incubate at 37°C and check after 24 and 48 h for the presence of colonies (see Note 9).

13. Place 200 µL of the control and the exposed conidia or spores in microtitration plates and incubate at 37°C or 48 h in a spectrophotometer reader that is able to monitor the growth automatically.

14. Always microscopically examine the controls and exposed conidia or spores to ensure that there are not differences in morphology between them. Pay attention that in the case of presence of PAFE, the difference is the time at which the germination starts; no changes in morphology should be present during the exposure. In addition, the experiments have to be performed in duplicate or triplicate (see Note 8).

### 3.3. Quantification of PAFE for Yeasts

1. PAFE is calculated by taking the difference in time required for the control and the exposed yeasts to grow 1 log$_{10}$ after drug removal.

2. The formula $PAFE = T - C$ can be applied, for which, $T$ is the time at which the exposed yeasts growth and $C$ is the time at which the corresponding control growth and is expressed in hours (see Note 5).

### 3.4. Quantification of PAFE for Filamentous Fungi

1. It is recommended to use the point in the growth curve optical density (OD)$_0$. This point is calculated as the first increase in OD for three consecutive measure-
Postantifungal Effect

Fig. 1. Interpretive schema of growth curves of control and exposed conidia. Example: \( T = 12.5 \) h; \( C = 6.5 \) h. Postantifungal effect = \( T - C = 12.5 - 6.5 = 6 \) h.

1. Measurements for the control and the exposed conidia or spores for each strain at each tested condition.

2. PAFE is calculated using the formula: \( \text{PAFE} = T - C \), where \( T \) is the first increase in \( \text{OD}_0 \) of the drug exposed conidia or spores and \( C \) is the first increase in \( \text{OD}_0 \) to the corresponding control (see Fig. 1).

3. Calculate the mean, coefficient of variation, and upper-lower 95% confidence interval for those, control and exposed conidia or spores at each condition for at least 4–8 growth curves for each.

4. PAFE is considered to be present when the growth of the exposed conidia or spores is delayed and the lower 95% confidence interval is delayed until beyond the upper 95% confidence interval of the control. Otherwise, when regrowth of drug-exposure isolates occurred within the upper 95% confidence interval of the corresponding controls, PAFE is considered absent.

4. Notes

1. Drugs that are dissolved in DMSO should be diluted at least 50 times in the medium. If the stock solution is at concentration of 400 \( \mu \)g/mL, then the starting concentration will be 8 \( \mu \)g/mL. Calculate carefully the total volume that is necessary to evaluate all the strains with all the concentrations.

2. RPMI-1640 is the standard medium, but other media also can be used.

3. To evaluate PAFE, it is recommended first to determine the MIC of the strains to plan adequately the concentration at which the PAFE will be evaluated. This concentration might be equal to, greater than, or less than the MIC values, for instance 1, 4, 0.5× the MIC. Always explore the pharmacokinetic profile of the drug.

4. Note that the CFU methodology is described here for the determination of PAFE for yeasts; however, the spectrophotometer procedure also is suitable.
5. Washing and decanting of 98% of the supernatant was demonstrated to reduce the concentration of the drug as much as 10,000-fold (1). Some technical problems might be appear as precipitation of some drugs, especially in the case of AMB. In such cases other procedure than washing, as filtration, can be employed. Dilution is not recommended for the fact that the amount of conidia or spores might insufficient for the correct monitoring of fungal growth.

6. Tween-80 is recommended for Zygomycetes, whereas Tween-20 is recommended for the other filamentous genera, including Aspergillus, black fungi, and others.

7. If the drug concentration is four times the MIC with a MIC value of 4 µg/mL, the final concentration will be 16 µg/mL. Therefore, the final volume of 10 mL is: 1 mL of fungal suspension plus the calculated mL that contain 16 µg/mL and the rest to complete the volume is RPMI-1640 alone.

8. At this point, it is important to perform killing studies and microscopic evaluation, for instance, if after 4 h of incubation of Aspergillus with AMB or azoles, germination and killing are not observed (2). However, for Zygomycetes, it depends on the genera and the time of incubation (6).

9. For CFU determination, incubation varies depending on slow- or fast-growing fungi, from 12–24 h for Zygomycetes and to 96 h for black fungi. In addition, it is very important that the CFU of controls and exposed fungi remain in a narrow range, such 2 × 10⁴ to 4 × 10⁴ CFU/mL. The inconvenience is that if the range is wide, false-positive results can be present. In other words, the difference in time for growing between the control and exposed fungi can be caused by small amount of conidia or spores and not as result of the presence of PAFE.

References


Evaluation of Immunotherapy in Invasive Candidiasis

Antifungal Activity and Cytokine Expression Assays

Maria Simitsopoulou and Emmanuel Roilides

Summary

*Candida* albicans and non-albicans *Candida* spp. can cause serious infections in hospitalized and immunocompromised patients. A large number of antifungal agents and immunomodulators have been developed and may interact with both polymorphonuclear and mononuclear phagocytes against blastoconidia and pseudohyphae of *Candida* spp. Blastoconidia are predominantly destroyed by phagocytosis, whereas pseudohyphal killing is initiated after the attachment of phagocytes to the cell wall of the organism and subsequent extracellular release of oxidative and nonoxidative metabolites. An array of methods to evaluate phagocytosis of blastoconidia, oxidative burst, expression of cytokines and chemokines in response to *Candida* spp., as well as candidacidal activity are described in this chapter.

**Key Words:** *Candida* spp.; phagocytes; antifungal function; phagocytosis; cytokine expression.

1. Introduction

1.1. Invasive Candida Infections

Invasive candidiasis is one of the leading causes of morbidity and mortality in hospitalized and immunocompromised patients nowadays (1). Although *Candida albicans* has been historically the most frequent cause of invasive candidiasis, non-*albicans Candida* spp. are becoming increasingly important being more resistant to mainstream antifungal agents than *C. albicans* (2). Hence, the intrinsic host response against *Candida* spp. and the effects of the immunomodulatory cytokine network on it need to be re-evaluated, especially in conjunction with the newer antifungal agents.

*Candida* spp. replicate by cell-budding and grow as blastoconidia, the infectious form of the organism. When *C. albicans* and most other *Candida* spp. are
found in environments of optimal pH and temperature, blastoconidia can germinate to pseudohyphae, the invasive form of the fungus. The fungal cell wall, a major component of which are mannans, is the main target of the antifungal metabolites of host phagocytes.

1.2. Host Defense Against Candida

Clinical and experimental data have demonstrated that innate immunity based on intact antifungal activity of phagocytes is critical in the outcome of invasive candidiasis. Quantitative and qualitative modulation of anticandidal host defense using cytokines as adjuncts to antifungal drug therapy has been supported by extensive in vitro and in vivo preclinical data, as well as some limited clinical results (3). Host innate immune response to Candida spp. is predominantly based on the antifungal efficacy of cytokine-activated tissue macrophages (e.g., peritoneal macrophages, Kupffer liver cells, splenic macrophages) and circulating neutrophils (polymorphonuclear leukocytes [PMNs]), as well as monocytes (mononuclear cells [MNCs]).

1.2.1. Phagocytic Cells

Macrophages have lost the capacity to produce significant amounts of oxygen-dependent metabolites. In contrast, they possess potent phagocytic activity and nonoxidative antifungal mechanisms such as cationic proteins and antifungal enzymes. These cells ingest blastoconidia of Candida spp., but they poorly inhibit their germination to pseudohyphae if they are not activated by cytokines, such as interferon-γ. Thus, blastoconidia may escape the inhibitory activity of macrophages and germinate to pseudohyphae, which pierce the macrophage membrane exiting the cell.

Circulating phagocytes, that is, PMNs and MNCs, are summoned to the site of Candida invasion. Although PMNs are more numerous, both circulating phagocytic cell populations are highly active against blastoconidia and pseudohyphae. The killing of C. albicans blastoconidia by PMNs and MNCs occurs intracellularly after phagocytosis and inclusion in phagosomes, whereas the killing of pseudohyphae is initiated by attachment to their cell wall through glycoprotein ligands even without serum opsonization and the secretion of antifungal phagocyte products. The antifungal function is greatly dependent on functional nicotinamide adenine dinucleotide phosphate oxidase and oxygen-dependent, as well as nonoxygen-dependent antifungal products. These metabolites disrupt the ergosterol- and chitin-rich membrane of pseudohyphae causing loss of intracellular ions and cell death (4–6).

1.2.2. Cytokines

A number of cytokines and chemokines are expressed and secreted on Candida infection. Cytokines that are of most interest because of their ability to
upregulate the function of phagocytes are the hematopoietic growth factors granulocyte colony-stimulating factor, granulocyte-macrophage colony-stimulating factor, and macrophage colony-stimulating factor. In addition, cytokines of T-helper type-1, such as interferon-γ, interleukin (IL)-12, and IL-15, as well as tumor necrosis factor-α, are also of interest. Cytokines of T-helper type-2 such as IL-4 and IL-10 exert an overall suppressive effect on antifungal function of phagocytes against *Candida* spp.

### 1.3. Evaluation of Antifungal Function

The interaction of *Candida* spp. with phagocytes activates antifungal functional responses, of which the most widely studied are the following:

1. Phagocytosis of blastoconidia.
2. Oxidative burst of phagocytes (i.e., superoxide anion \(O_2^-\) production) on stimulation by blastoconidia or pseudohyphae.
3. Expression and secretion of cytokines and chemokines in response to the organism.
4. Destructive activity of phagocytes on the organism. This can be assessed as either intracellular growth inhibition or killing of blastoconidia or extracellular phagocyte-induced damage of pseudohyphae.

The methods to evaluate these functions of phagocytes during immunotherapy against invasive *Candida* infections are briefly described in this chapter.

### 2. Materials

#### 2.1. Blastocandida and Pseudohyphae of *C. albicans* and Other Candida spp.

1. Clinical isolates or strains from a culture bank (i.e., American Type Culture Collection, Centraalbureau voor Schimmelcultures, etc).
2. Mycological media including potato dextrose agar and Sabouraud dextrose (SD) agar.
3. Hanks’ balanced salt solution with calcium and magnesium (HBSS+) (Gibco BRL, Life Technologies Ltd., Paisley, Scotland, UK).
4. RPMI-1640 culture medium (CM) (Gibco BRL).

#### 2.2. Phagocytes

##### 2.2.1. Isolation of PMNs, Isolation of MNCs From Blood Buffy Coat (Leukocyte Layer), and Enrichment of MNCs by Adherence

1. Sterile 50-mL polypropylene conical tubes (Costar, Corning Inc., Corning, NY).
2. Sterile 10-mL disposable pipets.
4. Sterile water.
5. Heparin (1000 U/mL).
6. Hanks’ Balanced Salt Solution without calcium or magnesium (HBSS−).
7. 3% (w/v) Dextran T500 (Sigma Chemical Co, St. Louis, MO; cat. no. D5251) in HBSS\(^{-}\). Dissolve dextran in HBSS\(^{-}\) by swirling on a warm plate.
8. 3.5% (w/v) NaCl in H\(_2\)O.
9. Ficoll Histopaque-1077: lymphocyte separation medium from Gibco BRL (see Note 1).
10. Trypan blue (Sigma, St. Louis, MO).
12. CM with fetal calf serum: RPMI-1640 supplemented with 10% FCS, 100 U/mL of penicillin, and 100 \(\mu\)g/mL of streptomycin.
13. Tissue culture 25- or 75-cm\(^2\) plastic flasks and plates with 6, 12, or 24 wells (Costar, Corning Inc.).
14. 18-mm round glass cover slips (Thomas Scientific, Swedesboro, NJ; cat. no. 6662-Q43).

2.2.2. Isolation of Rabbit Kupffer Cells and Rabbit Splenic Adherent Cells

1. Sterile forceps and scissors.
2. Phosphate-buffered saline (PBS): 10 mM phosphate buffer, pH 7.4, 120 mM NaCl, 2.7 mM KCl (Sigma; cat. no. 1000-3).
3. 0.4 \(\mu\)g/mL collagenase I (Sigma; cat. no. C-1677).
4. 4 U/mL DNase I (MBI Fermentas, St. Leon-Rut, Germany).
5. Nylon wool fiber (pore size: 40 \(\mu\)M) columns (PolySciences Inc., Warrington, PA).
6. CM with pooled human serum (HS): 25% v/v HS in RPMI-1640 containing 100 U/mL of penicillin and 100 \(\mu\)g/mL of streptomycin.

2.3. Assessment of Fungal Cell Damage

2.3.1. Assessment of Hyphal Damage (MTT Assay)

1. RPMI-1640.
2. HBSS\(^{+}\) and HBSS\(^{-}\) without phenol red.
3. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; 10X): 5 mg/mL (Sigma; cat. no. M2128).
4. Acidic isopropanol: 95 mL isopropanol + 5 mL 1 N HCl.
5. 0.5% sodium deoxycholate.

2.3.2. Assessment of Oxidative Burst (O\(_2^-\) Assay)

1. Sterile 15-mL polypropylene tubes.
2. Microplate with 96 flat-bottom wells.
3. RPMI-1640.
4. HBSS\(^{+}\) without phenol red.
5. Pooled HS derived from healthy donors and stored at –70\(^{\circ}\)C.
6. 1.2 mM cytochrome c (horse heart; Sigma; cat. no. C4186).
7. 1 mM N-formyl-methionyl-leucyl-phenylalanine (FMLP; Sigma; cat. no. F3506).
8. 10 \(\mu\)g/mL phorbol myristate acetate (PMA; Sigma; cat. no. P-8139).
9. 1 mg/mL superoxide dismutase (SOD; Sigma; cat. no. S-8524).
2.3.3. **Phagocytosis Assay With PMNs and MNCs**

1. 18-mm Round glass cover slips (see **Subheading 2.2.1.**, step 14).
2. Pooled HS for opsonization.
3. CM-HS (see **Subheading 2.2.2.**, step 6).
4. HBSS⁺.
5. May–Grunwald/Giemsa stain.
6. 75% Ethanol.

2.3.4. **Candidacidal Assay (Growth Inhibition of Blastocendidia by PMNs or MNCs)**

1. Sterile 2-mL screw-cap polypropylene tubes (Costar, Corning Inc).
2. Pooled HS or bovine serum albumin (Sigma).
3. HBSS⁺ and HBSS⁻.
4. Sterile water.
5. Petri dishes with SD agar.
6. Sterilized glass rods for smearing on the surface of agar.

2.4. **Assessment of Cytokine and Chemokine Production Using Reverse Transcription Polymerase Chain Reaction**

2.4.1. **Isolation of Total RNA**

1. Sterile 1.5-mL snap cap tubes.
2. TRIzol lysis reagent: a mixture of phenol and guanidine isothiocyanate (Invitrogen Inc., Carlsbad, CA).
3. Chloroform.
4. Isopropanol.
5. Diethylpyrocarbonate (DEPC)-treated sterile water.
6. TE buffer: 50 mM Tris-HCl, 10 mM ethylenediaminetetraacetic acid (EDTA), pH 7.5.

2.4.2. **Reverse Transcriptase Polymerase Chain Reaction**

1. Sterile thin-walled 0.2-mL polymerase chain reaction (PCR) tubes.
2. Target primers (TIB MOLBIOL).
3. Internal control primers (TIB MOLBIOL).
4. Reverse transcriptase (RT)/Platinum Taq mix (Invitrogen).
5. 2X reaction mix containing 0.4 mM of each deoxynucleotide 5’-triphosphates, 2.4 mM MgSO₄ (Invitrogen).

2.5. **Assessment of Cytokine and Chemokine Production Using Enzyme-Linked Immunosorbent Assay**

2.5.1. **Isolation of Intracellular Extract**

1. PBS.
2. Lysis buffer: 25 mM HEPES, pH 7.8; 0.5% nonident-P40; 0.1% sodium dodecyl sulfate (SDS); 0.5 M NaCl; 5 mM EDTA; 0.1 mM sodium deoxycholate; 1 mM
phenylmethylsulphonyl fluoride; 0.1 mg/mL aprotinin; 0.1 mg/mL leupeptin; 0.1 mg/mL pepstatin.

2.5.2. Enzyme-Linked Immunosorbent Assay

1. Murine monoclonal antibody specific for the protein of interest.
2. 25X wash buffer solution of buffered surfactant (R&D Systems, Minneapolis, MN).
3. Polyclonal antibody specific for the protein of interest conjugated to horseradish peroxidase.

3. Methods

3.1. Preparation of Blastocnidia and Pseudohyphae of C. albicans and Other Candida spp. (see Note 2)

1. Take a small aliquot of Candida stock maintained in a solution containing 25% glycerol and 75% peptone at –20°C.
2. Inoculate an SD agar plate and incubate at 37°C overnight.
3. The next day, pick two to three colonies from the plate and scrape them off the agar surface with a sterile cotton swab.
4. Suspend them in a 50-mL polypropylene conical tube and incubate in a rotating water bath at 37°C overnight.
5. Next morning, centrifuge the tube at 400 g for 10 min.
6. Wash pellet once with HBSS$^+$ and resuspend in HBSS$^+$.
7. Determine the concentration of suspension by counting blastocnidia on a hemocytometer.
8. In order to obtain pseudohyphae, make a suspension of 1–2 × 10$^5$/mL blastocnidia in RPMI-1640 and incubate at 37°C for appropriate times (see Notes 3 and 4).

3.2. Phagocytes

3.2.1. Isolation of PMNs

1. In a 50-mL polypropylene conical tube, place heparinized (10 U/mL, heparin) blood and add half volume of 3% Dextran T500 solution. Mix carefully by gentle inversion. Take care not to introduce bubbles during mixing to avoid cell membrane damage.
2. Incubate the homogeneous solution at room temperature for 20 min to allow the formation of two layers. The lower layer contains the sedimentary erythrocytes and the upper layer the leukocytes.
3. Transfer the top layer to a new 50-mL tube and underlayer 13 mL of Ficoll (Histopaque), inserting the tip of the pipet at the bottom of the tube and slowly releasing the solution to create a distinct interface between the two layers. Alternatively, in a 50-mL tube, add 13 mL of Ficoll first and overlay the leukocyte
suspension of step 2. This can be facilitated by tilting the tube that contains Ficoll so that the leukocyte suspension trickles down the side of the tube. At the end, Ficoll is at the bottom and leukocytes at the top. It is essential that both Ficoll and leukocyte suspension are at room temperature and there is a district interface between Ficoll and leukocyte suspension.

4. Centrifuge at 400g at room temperature for 20 min. After centrifugation, PMNs and remaining erythrocytes are accumulated in the pellet and MNCs are collected on a cloudy layer above the Ficoll layer. Above MNC layer there is a supernatant containing plasma and platelets (Fig. 1).

5. Sequentially aspirate all three layers using a 21-gage needle connected to an aspirator with a fluid trap and leave PMN-containing pellet intact. Take particular care to completely remove MNCs before aspirating Ficoll.

6. Lyse the erythrocytes hypotonically by suspending the pellet in 1 mL of sterile water. Dissolve pellet by swirling for 20 s and immediately add one third volume of 3.5% NaCl to prevent lysis of PMNs.

7. Add 40 mL of HBSS− and centrifuge at 400g for 10 min.

8. Suspend the pellet in 1 mL HBSS− and count the number of viable PMNs per milliliter on a hemocytometer after mixing with trypan blue.

9. Trypan blue staining procedure and cell counting: dilute PMNs 1:50 in HBSS− (i.e., 20 µL plus 980 µL of HBSS−) and mix 20 µL of diluted PMNs with 20 µL of trypan blue solution. Place 20 µL of the mixture on a hemocytometer and count the cells. Blue cells are dead; cells excluding the stain are alive. If the cell count on the hemocytometer is $\alpha$, the actual cell count in the suspension is $\alpha \times 10^6$/mL.

3.2.2. Isolation of MNCs From Whole Blood or Buffy Coat (Leukocyte Layer)

1. If you start from whole blood, follow the isolation procedure of Subheading 3.2.1. up to step 4. If you start from buffy coat, dilute it with equal volume of HBSS−, and go directly to step 4 of Subheading 3.2.1. After centrifugation, the plasma, mononuclear cells (mainly lymphocytes and monocytes) and Ficoll form three layers above the PMN-remaining erythrocyte pellet (from top to bottom) (Fig. 1).

2. Aspirate and discard the top layer (plasma).

3. Transfer the MNC layer to a clean 50-mL tube with a sterile plastic pipet and add up to 40 mL of HBSS−.

4. Centrifuge at 400g for 10 min to pellet the cells.

5. Aspirate the supernatant and resuspend pellet in HBSS−.

6. Determine the cell concentration by Trypan blue staining (see Subheading 3.2.1., step 9).

7. Estimate the percentage of MNCs within the MNC population by modified May–Grunwald/Giemsa stain procedure as follows:
   a. Smear the cell suspension onto a microscopic slide.
   b. Immerse slide in May–Grunwald stain for 10 min and in Giemsa stain for 30 min.
Fig. 1. Isolation of polymorphonuclear leukocytes and mononuclear cells from whole blood or buffy coat. PMNs, polymorphonuclear leukocytes; MNCs, mononuclear cells.
c. Rinse with water and dry. Calculate the percentage of MNCs out of a total 100 cells.

### 3.2.3. Enrichment of MNCs by Adherence

1. Place the MNCs in a 12-well culture plate at an estimated concentration of $1–2 \times 10^6$ MNCs per milliliter of CM. Use appropriate numbers of cells and volumes of medium if you use culture plates with different number of wells. For phagocytosis assays, place alcohol-sterilized 18-mm round glass cover slips in the center of each well of a 12-well plate. Carefully place 0.2 mL of a MNC suspension with $5 \times 10^6$ MNCs per milliliter on each cover slip.
2. Incubate at 37°C for 2 h (MNCs adhere on plastic or glass surfaces).
3. Wash twice with warm HBSS–. Nonadherent T lymphocytes and many B lymphocytes are removed; the remaining adherent cells are enriched MNCs.
4. Add 20 mL of cold HBSS– and gently scrape whole surface of the flask.
5. Centrifuge at 400 g for 10 min, aspirate the supernatant, and resuspend the pellet of enriched MNCs.

### 3.2.4. Isolation of Rabbit Kupffer Cells

1. Incise portal veins to allow efflux of perfusate into the liver.
2. Perfuse liver with PBS several times and once with PBS warmed to 37°C and containing 0.4 mg/mL of collagenase and 4 U/mL DNase I.
3. After excision, cut liver into small pieces and homogenize using sterile polyethylene bags.
4. Combine cell homogenate and perfusate and centrifuge five times at 100 g and 4°C for 5 min to separate Kupffer cells (KCs) from the more abundant hepatocytes. The latter cells are particularly large and are pelleted, whereas KCs are smaller and stay in the supernatant during the 5-min low-speed centrifugations.
5. Suspend KCs in HBSS– and count them by Trypan blue staining.

### 3.2.5. Isolation of Rabbit Splenic Adherent Cells

1. Excise spleens, make several incisions to the splenic capsule and homogenize organs in polyethylene bags with PBS containing 0.4 µg/mL collagenase.
2. Incubate homogenate at 37°C for 8–10 min and pass cell suspension through a prewarmed 40-µM nylon wool fiber column.
3. Incubate at 37°C for 1 h and elute the nylon-adherent cells with three volumes of CM. Splenic adherent cells (SACs) can be further purified by seeding in culture flasks.

### 3.3. Assessment of Fungal Cell Damage

#### 3.3.1. Damage of Pseudohyphae: MTT Microassay

MTT is a tetrazolium dye used to quantify phagocyte-mediated damage to *Candida* pseudohyphae. MTT is reduced to a blue-colored formazan deriva-
Disruption of the dehydrogenase activity is affected by pseudohyphal damage after incubation with phagocytes. Percent hyphal damage induced is assessed colorimetrically.

1. Make a suspension of $2.5 \times 10^4$/mL blastoconidia in RPMI-1640.
2. Add 200 µL of suspension with blastoconidia per well in a 96-well flat-bottomed plate and incubate at 37°C for appropriate times (see Notes 3 and 4).
3. Aspirate RPMI-1640 from each well and immediately add the required volume of HBSS+ containing the effector cells (MNCs or PMNs) at desired effector cell to target (E:T) ratios (Note: to avoid drying of the hyphae aspirate one row at a time and immediately add indicated volume of HBSS+).
4. Incubate at 37°C for 2 h.
5. Aspirate contents from each well one row at a time, taking care not to disturb the lawn of hyphae covering the bottom of each well.
6. Add 60 µL of 0.5% sodium deoxycholate.
7. Centrifuge microplate at 800 g 15°C for 5 min.
8. Aspirate and add 200 µL of sterile water.
9. Centrifuge microplate at 800 g 15°C for 5 min.
10. Repeat the washing step twice.
11. After the third wash and centrifugation steps, aspirate and add 200 µL of 1X MTT solution (0.5 mg/mL in RPMI without phenol red).
12. Incubate at 37°C and 5% CO₂ for approx 3 h.
13. Centrifuge microplate at 15°C and 800g for 5 min.
14. Aspirate the wells dry. At this point, the plate can be stored for subsequent reading at −20°C overnight.
15. Add 120 µL of acidic isopropanol to each well. Swirl microplate to obtain a homogeneous solution.
16. Transfer 100 µL from each well to a 96-well plate.
17. Measure absorbance at 450 nm with reference wavelength set at 690 nm. Include a blank well containing 100 µL of acidic isopropanol.
18. % Hyphal damage = $1 - (X/C) \times 100$, where X is the absorbance of test wells and C is the absorbance of control wells with hyphae only.

3.3.2. Damage of Pseudohyphae: MTT Macroassay

1. Prepare a suspension of $2.5 \times 10^5$ C. albicans blastoconidia per mL in RPMI-1640 and plate 1 mL aliquots in each well of a 24-well plate.
2. Incubate the plate at 37°C for 4 h (C. albicans) or appropriate times depending on Candida spp. to allow germination of blastoconidia into hyphae (see Notes 3 and 4).
3. Aspirate RPMI-1640 from each well and immediately add the required volume of HBSS+ containing the effector cells (PMNs or MNCs) at desired E:T ratios.
4. Incubate at 37°C for 2 h.
5. Aspirate supernatants from each well and lyse PMNs or MNCs by adding 300 µL of 0.5% sodium deoxycholate.
6. Aspirate and wash hyphae with 1 mL of sterile water three times.
7. After the third wash, add 1 mL of RPMI-1640 containing 1X MTT solution (0.5 mg/mL in RPMI) and incubate at 37°C and 5% CO₂ for 3 h.
8. At the end of the incubation, aspirate the wells dry and extract MTT dye by adding 200 μL of acidic isopropanol to each well.
9. Transfer 150 μL from each well to a 96-well plate.
10. Measure absorbance at 450 nm with reference wavelength set at 690 nm. Include a blank well containing 150 μL of acidic isopropanol.
11. Calculate % hyphal damage as performed with the MTT micromethod (see Subheading 3.3.1., step 18).

3.3.3. Superoxide Anion Microassay

The O₂⁻ production by phagocytes in response to fungal stimulation is assessed as reduction of ferricytochrome c. The rate of reduction is expressed in nM O₂⁻/10⁶ phagocytes/1 h using 29.5 × 10⁴ M⁻¹ cm⁻¹ as the extinction coefficient for reduced cytochrome c at 550 nm. The bacterial chemoattractive tripeptide FMLP or PMA can be incubated with PMNs as soluble stimuli of O₂⁻ production. The enzyme, SOD, converts O₂⁻ into H₂O₂ at neutral or alkaline pH and thus the phagocyte nicotinamide adenine dinucleotide phosphate-specific reduction of ferricytochrome c is prevented.

1. Dilute suspension of blastoconidia in RPMI-1640 to a final concentration of 10⁶ per milliliter.
2. Add 200 μL of blastoconidial suspension per well in a 96-well flat-bottomed plate and incubate at 37°C for 4 h (C. albicans) or appropriate times depending on the Candida spp. to allow germination of blastoconidia into hyphae (see Notes 3 and 4).
3. In wells where O₂⁻ production in response to non-opsonized hyphae is assessed, aspirate and replace RPMI-1640 with 100 μL HBSS⁺ (see Note 5).
4. In order to opsonize pseudohyphae, add 100 μL 50% pooled HS in wells. Incubate at 37°C for 30 min.
5. Wash twice with HBSS⁺.
6. Add 50 μM ferricytochrome c and phagocytes at required E:T ratio with opsonized or non-opsonized hyphae in wells. An E:T ratio of 1:1 or 2:1 is frequently used and is sufficient to detect differences in the amounts of reduced cytochrome c in response to opsonized vs non-opsonized hyphae (see Note 6).
7. Assess O₂⁻ production by PMNs or MNCs to soluble stimuli adding 0.5 or 1 μM FMLP or 50 ng/mL PMA and cytochrome c in wells with PMNs alone. Wells with PMNs or MNCs and cytochrome c in HBSS⁺ are used to determine baseline O₂⁻ production.
8. Incubate at 37°C on a rotating rack for 1 h. Transfer 100 μL to a new 96-well plate and read absorbance (Abs) at 550 nm with a reference wavelength set at 690 nm. Take absorbance values using as blank 100 μL cytochrome c. The extinction coefficient of reduced cytochrome c used to calculate the O₂⁻ production is 29.5 × 10⁴ M⁻¹ cm⁻¹.
9. In some wells, add 50 $\mu$g/mL SOD, phagocytes, ferricytochrome c, and a stimulus. Any absorbance read in the SOD wells is nonspecific and should be subtracted from the values obtained in the assay wells.

3.3.4. Superoxide Anion Macroassay

1. Opsonize blastoconidia by addition of 1 mL pooled HS to 1 mL of a suspension containing $10^8$ blastoconidia and by incubation at 37°C for 30 min.
2. Wash twice with HBSS+ and suspend blastoconidia in an appropriate volume of HBSS+.
3. Transfer $10^6$ phagocytes to sterile 2-mL screw-cap tubes.
4. Add $2 \times 10^6$ serum-opsonized blastoconidia (E:T ratio 1:2) and 50 $\mu$M cytochrome c in 1 mL final volume of HBSS+. Include control tubes to assess $O_2^-$ production by phagocytes to soluble stimuli adding 0.5 or 1 $\mu$M of FMLP or 50 ng/mL of PMA in tubes containing $10^6$ phagocytes and cytochrome c. Incubate experimental and control tubes at 37°C for 15 min on a rotating rack.
5. Transfer 100 $\mu$L to an enzyme-linked immunosorbent assay (ELISA) 96-well microplate and read Abs at 550 nm (see Subheading 3.3.3., step 8).

3.3.5. PMN Phagocytosis Assay

1. Incubate $10^8$ Candida blastoconidia in 50% pooled HS at 37°C for 30 min in a 15-mL glass tube (2-mL final volume) for opsonization.
2. Wash the blastoconidia twice and resuspend in HBSS+ at a concentration of $10^7$/mL. Keep samples on ice until use.
3. In a 2-mL screw-cap tube, add 100 $\mu$L of suspension containing $10^6$ PMNs, 100 $\mu$L of suspension containing $10^6$ blastoconidia, and 800 $\mu$L of HBSS+
4. Incubate mixture at 37°C on a shaker for 15 min.
5. Transfer 200 $\mu$L to a new tube, centrifuge at 400g for 5 min, and smear pellet on a microslide.
6. Fix and stain the slides according to May–Grunwald/Giemsa stain procedure (see Subheading 3.2.2.).
7. Observe and count phagocytosed blastoconidia as in Subheading 3.3.6.

3.3.6. MNC Phagocytosis Assay

1. Keep the cover slips in 75% ethanol and flame briefly before use. Place sterile round cover slips into the wells of a 12-well tissue culture plate.
2. On each cover slip, distribute 200 $\mu$L of a suspension of $5 \times 10^6$ MNCs/mL in CM-HS evenly. Avoid spillage on the surface of the well.
3. Incubate at 37°C for 2 h to allow MNC adherence on the glass cover slips.
4. Wash each cover slip with 1 mL of warm HBSS+ twice. Adherent MNCs will remain, whereas nonadherent lymphocytes will be washed off (Note: HBSS+ must be prewarmed to 37°C).
5. Add $10^6$ opsonized blastoconidia/milliliter in CM to each cover slip and incubate at 37°C for 15, 30, or 60 min depending on the Candida spp. and the goal of the study.
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6. Wash with 1 mL of warm HBSS−, remove, and air-dry cover slips.
7. Fix and stain the cover slips according to May–Grunwald/Giemsa stain procedure (see Subheading 3.2.2.).
8. Mount cover slips on microscopic slides with permount.
9. Observe and count phagocytosed conidia under a light microscope. Distinguish between those that have a complete phagosome membrane around them or are ingested by more than 50% in a deepening of phagocytic membrane. Do not count blastoconidia that are attached or ingested by less than 50%.
10. Calculate the number of conidia phagocytosed/100 MNCs

\[
\text{Percentage of phagocytosis} = 100 \times \frac{B + C + D + E + F}{A + B + C + D + E + F}
\]

\[
\text{Phagocytosis index} = \frac{B + 2C + 3D + 4E + 5F}{B + C + D + E + F}
\]

where \(A\) is the number of cells that phagocytosed no conidia, \(B\) is the number that phagocytosed one conidium, \(C\) is the number that phagocytosed two conidia, \(D\) is the number that phagocytosed three conidia, \(E\) is the number that phagocytosed four conidia, and \(F\) is the number that phagocytosed more than four conidia.

3.3.7. Candidacidal Growth Inhibition Of Blastocnidia by PMNs or MNCs

1. Transfer 10^6 MNCs that had been pre-incubated with CM in 25 cm^2 tissue culture flasks or equal number of PMNs to sterile 2-mL screw-cap polypropylene tubes.
2. Add 10^6 blastoconidia suspended in HBSS+ and 10% pooled HS in a final volume of 1 mL. Include control tubes containing blastocnidia, pooled HS, HBSS+ but not MNCs or PMNs. Alternatively, add serum-opsonized blastocnidia (see Subheading 3.3.4.) and MNCs or PMNs in HBSS+ containing 0.1% bovine serum albumin.
3. Take a 0.1 mL aliquot from a “dedicated” tube at time 0, dilute it as in steps 5 and 6 and plate it on SD agar plates. These plates will give the count of the colony-forming units (CFUs) of the initial inoculum.
4. Incubate the tubes at 37°C for 30, 60, 90, or 120 min using a rotating rack to ensure constant mixing. The time depends on the goal of the study. More than one time point is desired.
5. Lyse MNCs or PMNs hypotonically by adding 0.1 mL of the mixture to 0.9 mL of sterile water. Vortex.
6. Take 0.1 mL of the suspension and add to 9.9 mL of HBSS−. Vortex well.
7. Plate 0.1 mL of diluted samples onto SD agar plates and incubate at 37°C for 18 h.
8. Count the colonies and calculate the candidacidal activity using the formula:
9. \(
\text{% growth inhibition} = (1 - X/C) \times 100, \text{ where } X \text{ is the number of CFUs with MNCs or PMNs at the end of incubation and } C \text{ is the number of CFUs without MNCs or PMNs at the end of incubation.}
\)
3.4. Assessment of Cytokine and Chemokine Production by MNCs Using RT-PCR

Immunomodulators expressed in phagocytes in response to Candida infection can be detected and quantified either at the ribonucleic acid (RNA) level by RT-PCR or at the protein level by the quantitative sandwich enzyme immunosorbent assay (Fig. 2). The method of RT-PCR described in Subheading 3.4.2. combines both complimentary deoxyribonucleic acid (cDNA) synthesis and PCR in a single tube using gene-specific primers and the polymerase mixture: Superscript II H–RT and Platinum Taq polymerase. PCR products are quantitated including a second set of internal control primers, which amplify a housekeeping gene in the RT-PCR (7–9). The amount of immunomodulatory mRNAs are expressed as a ratio between the sample mRNA and the internal control mRNA (10).

3.4.1. Isolation of Total RNA

1. Isolate MNCs by plastic adherence (follow protocols in Subheadings 3.2.2. and 3.2.3.).
2. Discard culture supernatant and add 1 mL of PBS.
3. Scrape cells off the wells of culture plate and transfer solution to 1.5-mL snap cap tubes.
4. Pellet cells by centrifugation at 400g at 4°C for 5 min and lyse cells by adding 1 mL of TRIzol reagent.
5. Homogenize solution pipeting carefully up and down several times.
6. Incubate the samples at room temperature for 5 min to allow dissociation of nucleoprotein complexes.
7. Add 0.2 mL of chloroform per 1 mL of TRIzol reagent.
8. Mix samples by hand vigorously for 15 s and incubate at room temperature for 5 min.
9. Centrifuge samples at 12,000g at 4°C for 10 min.
10. After centrifugation, the mixture separates into a lower red, phenol-chloroform phase, and a colorless upper aqueous phase. RNA is in the aqueous phase. Transfer the aqueous phase to another snap cap tube and precipitate RNA by adding 0.5 mL of ice-cold isopropanol per 1 mL of TRIzol reagent.
11. Incubate at room temperature for 10 min.
12. Centrifuge samples at 12,000g at 4°C for 15 min.
13. Remove the supernatant, wash the RNA pellet with 1 mL of ice-cold, 75% DEPC-treated ethanol.
14. Mix samples by vortexing and centrifuge at 9000g at 4°C for 5 min.
15. Decant ethanol and dry the RNA pellet at room temperature for 3 min. Do not dry the RNA pellet completely because it will not redissolve.
16. Dissolve RNA in 400–500 µL of RNase-free DEPC-treated deionized H₂O or TE buffer.
Fig. 2. Flow chart for detection of cytokines/chemokines expressed in phagocytes in response to *Candida* infection. RNA, ribonucleic acid; RT-PCR, reverse transcriptase-polymerase chain reaction; ELISA, enzyme-linked immunosorbent assay.

17. Quantify the amount of total RNA yield spectrophotometrically at 260 nm and use 0.1–1 µg RNA for RT-PCR. The amount of template used depends whether the RNA species for detection is expressed at high or low levels.

### 3.4.2. RT-PCR of MNC Total RNA

1. Use thin-walled 0.2-mL PCR tubes and add the following reagents *(see Notes 7 and 8)*:

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Volume</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>2X reaction mix</td>
<td>25 µL</td>
<td>1X</td>
</tr>
<tr>
<td>Forward primer: 10 µM</td>
<td>X µL</td>
<td>0.2 µM</td>
</tr>
<tr>
<td>Reverse primer: 10 µM</td>
<td>X µL</td>
<td>0.2 µM</td>
</tr>
<tr>
<td>Internal control forward primer: 5 µM</td>
<td>X µL</td>
<td>0.1 µM</td>
</tr>
<tr>
<td>Internal control reverse primer: 5 µM</td>
<td>X µL</td>
<td>0.1 µM</td>
</tr>
<tr>
<td>RT/platinum Taq polymerase mix</td>
<td>1 unit</td>
<td></td>
</tr>
<tr>
<td>Template: 0.01–1 mg total RNA</td>
<td>X µL</td>
<td></td>
</tr>
<tr>
<td>DEPC-treated water</td>
<td>to 50 µL</td>
<td></td>
</tr>
</tbody>
</table>
2. To rule out contamination of genomic DNA in RNA preparation, include a negative RT-PCR control tube containing all reagents except the polymerase mix. Replace the RT/platinum Taq polymerase mix with 2 U of Taq DNA polymerase (see Note 9).

3. The following cycling conditions for cDNA and double-stranded DNA synthesis are applicable for Perkin–Elmer Thermal Cycler 9600.
   a. 1 cycle: 48°C for 30 min (cDNA synthesis).
   b. 94°C for 2 min (inactivation of RT, reactivation of Taq polymerase).
   c. 35 cycles: denaturation step: 94°C for 15 s.
   d. Annealing step: 50–60°C.
   e. Extension step: 68°C for 1 min.
   f. 1 cycle: 68°C for 7 min (final extension), 4°C ∞.

4. Detect PCR products on 1.2% agarose gel by electrophoresis and ethidium bromide staining (see Note 10).

5. Quantitate PCR products using a CCD camera to capture the DNA image and a computer software program to express the band intensity into nanograms/milliliter (see Note 11).

### 3.5. Assessment of Cytokine and Chemokine Production by MNCs Using ELISA

The sandwich type of ELISA requires a monoclonal antibody that is specific for the protein to be detected and a polyclonal antibody conjugated to horseradish peroxidase. The test sample is sandwiched between the antibodies bound by noncovalent interactions. The protein is detected by the addition of equal amounts of hydrogen peroxide and tetramethylbenzidine, the substrate on which the enzyme acts to produce a color change (12,13). The absorbance of each sample is determined at 450 nm.

#### 3.5.1. Isolation of Intracellular Extract

1. Isolate MNCs by plastic adherence (follow protocols in Subheadings 3.2.1. and 3.2.3.).
2. Save culture supernatant to measure immunomodulatory proteins secreted extracellularly in response to Candida infection.
3. Wash once with 1 mL of PBS and lyse attached MNCs by adding 1 mL of freshly prepared lysis buffer to each well of culture plate.
4. Detach remaining MNCs by scraping the wells of culture plate and transfer solution to 1.5-mL snap-cap tubes. Keep samples on ice.
5. Vortex samples vigorously for 30 s and pellet solubilized cell fragments by centrifugation at 12,000g, 4°C for 10 min.
6. Combine culture supernatants with the corresponding intracellular extracts to measure the total amount of specific protein expressed. Alternatively, one can treat culture supernatants and intracellular extracts separately for protein quantitation.
7. Keep samples at –20°C until processed by ELISA using the appropriate Quantikine kits depending on the cytokines under investigation. Avoid repeated freezing and thawing of samples.

3.5.2. Enzyme-Linked Immunosorbent Assay

General guidelines to consider for quantitation of proteins using the Quantikine kits supplied by R&D Systems are listed:

1. To quantitate the expression levels of a specific protein, the measured amount should fall within the concentration range of the standards provided in the kit. Reserve two ELISA strips for a trial experiment where the wells of one of the strips will contain various dilutions of the sample and the second strip will be used for the standards.
2. Dilutions of standards and samples should be made with utmost accuracy and consistency.
3. Before reading the microplate, make sure the color change appears uniform in all wells by passing the contents of each well several times through a multichannel pipet.
4. Convert the absorbance readings into picograms per milliliter of measured protein using a computer software program.

4. Notes

1. Histopaque-1077 contains polysucrose and sodium diatrizoate at a density of 1.077 g/mL.
2. Candida glabrata does not produce pseudohyphae.
3. Each Candida spp. needs a specific incubation time for germination: 4 h for C. albicans, 6 h for Candida tropicalis, and 8 h for Candida parapsilosis.
4. Pseudohyphal formation and microplate confluence is determined microscopically.
5. Cytochrome c can be stored in stock solution frozen at –70°C but must be freshly diluted for every assay.
6. Hanks’ balanced solution used to perform the superoxide anion assay should not contain phenol red because it adds to color intensity of ferrocytochrome c produced giving erroneously high Abs values.
7. It is recommended that the cDNA synthesis be carried out in RNase-free conditions. Pipet tips, tubes, laboratory glassware, or plastic-ware should be treated as follows:
   a. Fill glass beakers, bottles and cylinders with 0.1% DEPC.
   b. Incubate overnight at 37°C and then autoclave.
   c. Use baked/sterile spatulas to weigh solid reagents.
   d. Prepare DEPC-treated water and with this make 75% ethanol.
   e. Sterile disposable plasticware is essentially free of RNases and can be used without further treatment.
   f. Wear gloves to prepare solutions and change gloves frequently when working with RNA. Set aside items of glasses and plasticware treated with DEPC,
mark them and store them in a designated place for RNA WORK ONLY.
DEPC reacts with amines and cannot be used to treat solutions containing buffers with Tris-HCl. It is a suspected carcinogen. Handle with care.
8. Internal control RNAs to be used in RT-PCR are glyceraldehyde-3-phosphate dehydrogenase or aldolase A. The first RNA transcript is used for normalization of highly expressed transcripts, whereas aldolase A messenger (m) RNA can be used to quantitate low abundant mRNAs. Avoid using β-actin as internal control for RT-PCR.
9. If genomic contamination is observed, treat RNA preparation with DNase I: Add the following to a sterile snap cap tube:
   a. 1 µg of RNA.
   b. 1 µL of 10X DNase buffer.
   c. 1 U amplification-grade DNase I.
   d. Bring volume up to 10 µL with DEPC-treated sterile H₂O.
   e. Incubate at room temperature for 15 min and terminate the reaction by adding f. 1 µL of 25 mM EDTA and heating for 10 min at 65°C.
   g. Place sample on ice for 1 min and use this mixture for RT-PCR.
10. If cDNA synthesis is prepared separately and RT-PCR is performed consequently, the approximate length and the amount of the cDNA synthesis products are determined by neutral agarose gel electrophoresis followed by ethidium bromide staining. At neutral pH, the cDNA:RNA hybrid has similar electrophoretic mobility with double-stranded DNA.
11. If there is competition observed between the target PCR product and the co-amplified internal control, add MgSO₄ to a final concentration of 3 mM.

References
Interaction of *Histoplasma capsulatum* With Human Macrophages, Dendritic Cells, and Neutrophils

Simon L. Newman

Summary

*Histoplasma capsulatum* (Hc) is a dimorphic fungal pathogen indigenous to the Ohio and Mississippi River Valleys in the United States. Infection is initiated by inhalation of microconidia or small mycelial fragments into the terminal bronchioles of the lung. The conidia are taken up by alveolar macrophages (Mφ), in which they convert to the pathogenic yeast phase. The yeasts replicate in the alveolar Mφ and other Mφ recruited to the lung as part of the inflammatory response. Thus, the yeasts are able to disseminate from the lung to other organs, such as the liver and spleen. As a facultative intracellular parasite, the interaction of Hc yeasts with Mφ is a critical component of the host response to infection. In addition, Hc yeasts have critical interactions with inflammatory neutrophils, and with dendritic cells (DCs) in the lung and other organs. Indeed, recent new evidence suggests that DCs may be the key antigen-presenting cells that initiate cell-mediated immunity. Thus, the methods described in this chapter cover quantitation of the binding, ingestion, and intracellular replication of Hc yeasts in human Mφ, DCs, and neutrophils.

**Key Words:** *Histoplasma capsulatum*; macrophage; dendritic cell; neutrophil; phagocytosis.

1. Introduction

When phagocytic cells interact with a microbial pathogen, there are three key events that define the interaction: recognition; ingestion; and killing or intracellular replication, depending on the particular pathogen. Because *Histoplasma capsulatum* (Hc) is a facultative intracellular pathogen, it replicates in macrophages (Mφ) until they have been activated by T-cell cytokines (1,2). In contrast, human dendritic cells (DCs) kill ingested Hc yeasts (3), and neutrophils are able to inhibit the growth of yeasts but do not kill them (4). The methods described herein provide for the study of these three key interactions with human blood cells. Major procedures are described for the preparation of...
human monocyte-derived M\(\phi\), neutrophils, and DCs, and quantitation of the attachment, phagocytosis, and intracellular replication of Hc yeasts in these cells.

2. Materials

2.1. Solutions

1. 0.9% Sodium chloride (normal saline).
2. 3.8% Sodium citrate.
3. 6% Dextran in 0.9% saline.
4. 1 \(M\) CaCl\(_2\).
5. 0.01 \(M\) Phosphate-buffered saline (PBS).
6. PBSA: PBS containing 0.05% azide.
7. 1 mg/mL of trypan blue in PBS.
8. 0.5 \(M\) Carbonate–bicarbonate buffer, pH 9.0.
9. Hanks’ balance salt solution (HBSS)/HEPES: HBSS containing 20 \(mM\) HEPES and 10 \(\mu g/mL\) gentamicin.
10. HBSS/HEPES made with calcium- and magnesium-free HBSS.
11. HBSA: HBSS/HEPES containing 0.25% bovine serum albumin (BSA).
12. HBSA-AP, HBSA containing aprotinin (Sigma, St. Louis, MO) diluted 1/50.
14. 1% Paraformaldehyde in 0.01 \(M\) phosphate buffer, pH 7.2.

2.2. Media

1. For culture of adherent monocytes: M199 containing 10 \(\mu g/mL\) gentamicin and 10% autologous serum.
2. For culture of suspension monocytes: RPMI-1640 containing 10 \(\mu g/mL\) gentamicin and 12.5% autologous serum.
3. For intracellular growth assays: RPMI-1640 containing 10 \(\mu g/mL\) gentamicin and 5% fetal calf serum (FCS).
4. For growth of Hc in liquid media: Ham’s F12 plus 0.1 \(M\) glucose, 268 \(\mu M\) cysteine, 25 \(mM\) HEPES, and 6.8 \(mM\) glutamic acid, pH 7.5.
5. DC media: RPMI-1640 containing 10% FCS, 2 \(mM\) L-glutamine, 50 \(\mu M\) 2-mercaptoethanol, 1% pyruvate, 1% nonessential amino acids, and 50 \(\mu g/mL\) kanamycin.
6. 10X yeast nitrogen broth (Difco Laboratories, Detroit, MI). This broth is prepared 10-fold more concentrated than recommended by the manufacturer.

2.3. Kits

1. EasySep positive selection kit (cat. no. 18058) for human CD14+ cells (Stem Cell Technologies, Vancouver, Canada).
3. Methods

3.1. Maintenance, Growth, and Harvesting of Yeasts

3.1.1. Maintenance of Yeasts on Agar Slants
1. Hc yeasts, strain G217B, are maintained by passage twice weekly on BHI slants.
2. These slants are made per the manufacturer’s instructions.

3.1.2. Growth of Yeasts in Liquid Media
1. A small amount of yeasts are washed from a slant and grown in 50 mL of Ham’s F12 media for 48 h at 37°C in a shaking water bath set at 150 rotations per minute.

3.1.3. Harvesting of Yeasts
1. The yeasts are collected in a 50-mL centrifuge tube and spun at 384g for 10 min at 4°C.
2. Wash the yeasts twice in HBSS/HEPES, and then centrifuge at 84g for 7 min to remove large clumps of yeasts.
3. Count the yeasts on a hemocytometer and then standardize to the appropriate concentration in the desired media, which will depend on the particular assay being performed.

3.1.4. Heat-Killed Yeasts for Binding and Phagocytosis Assays
1. For preparing heat-killed (HK) yeasts, the low speed spin described above is omitted, and the yeasts are washed in PBS.
2. After the second wash, resuspend the yeasts in 50 mL of PBS and heat-kill at 65°C for 1 h.
3. Wash the yeasts twice more in PBS and sonicate to make a single cell suspension (see Note 1).
4. Standardize the yeasts to $2 \times 10^8$ /mL in PBSA and store at 4°C for up to 2 wk (see Note 2).

3.1.5. Fluorescent Labeling of Yeasts for Attachment and Phagocytosis Assays
1. Remove 0.5 mL of HK yeasts to a 1.5-mL microcentrifuge tube and centrifuge 1 min at 10,000 rpm in a microfuge (maximum speed).
2. Wash the yeasts twice with 1 mL of HBSA.
3. After the second wash resuspend the yeasts in fluorescein isothiocyanate (FITC) (0.01–0.1 mg/mL in carbonate–bicarbonate buffer) and incubate for 15–30 min at room temperature (RT) in the dark (see Note 3).
4. Centrifuge and wash twice in HBSA.
5. Resuspend the yeasts to the appropriate concentration in HBSA ($0.5–1 \times 10^7$/mL for the phagocytosis assay; $1 \times 10^7$/mL for the binding assay [5]).
3.2. Purification of Monocytes and Neutrophils From Human Blood

3.2.1. Purification of Monocytes

1. Thirty milliliters of freshly drawn blood is mixed with 3.3 mL of citrate in a 50-mL conical centrifuge tube (see Note 4).
2. Centrifuge the blood at 200 g for 20 min at RT.
3. Remove 10–15 mL of platelet-rich plasma (PRP) and centrifuge at 1800 g to remove the platelets. The platelet-poor plasma (PPP) obtained will be used to make the Ficoll–hypaque gradients.
4. Remove the rest of the PRP to a sterile glass bottle, and add 20 µL of CaCl₂ per mL of PRP.
5. Incubate at 37°C until a clot forms, and then at 4°C to retract the clot. The serum obtained will be used in preparing the culture medium.
6. Next, add 5 mL of warm dextran to each tube of blood, and dilute up to 50 mL with normal saline.
7. Pour the blood from each tube into another 50-mL conical tube and back again to mix.
8. Let the tubes sit 30 min at RT to settle out the erythrocytes.
9. Remove the top white cell layer from each tube to another 50-mL tube.
10. Fill each tube to the 50-mL mark, and then centrifuge at 246 g for 10 min at RT.
11. Pour off supernatant and resuspend each pellet in a mixture of 4 mL of PPP and 12 mL of saline.
12. Transfer 8 mL of each mixture to a 16 × 125-mm screw-cap tube.
13. Place a 9-inch sterile Pasteur pipet into each tube and underlayer the cells with 3 mL of Ficoll–hypaque by squirting the Ficoll–hypaque into the Pasteur pipet using a 10-mL syringe and a 21-gage needle.
14. Centrifuge at 500 g for 25 min at RT.
15. The mononuclear cells (MNCs) (monocytes and lymphocytes) will be at the interface of the PPP/saline mixture and the Ficoll–hypaque. The neutrophils are in the pellet with the erythrocytes. Remove the MNCs with a sterile transfer pipet to a 50-mL tube containing approx 20 mL of HBSS/HEPES.
16. Fill tube(s) to 50 mL with HBSS/HEPES and centrifuge at 384 g for 10 min at 5°C.
17. Wash the MNCs twice with cold Ca/Mg free HBSS/HEPES (to remove any platelets) and twice with cold regular HBSS/HEPES (centrifuge at 246 g). This is the stopping point for preparing monocytes for adherent culture into Mφ.

To obtain monocytes for suspension culture into Mφ, or for culture into DCs, a further purification is performed using the EasySep positive selection kit (Stem Cell Technologies, cat. no. 18058 ) for human CD14+ cells. When this purification is desired, the second two washes described in Subheading 3.2.1., step 17 with regular HBSS/HEPES are left out. Instead the cells are counted on a hemocytometer and the manufacturer’s instructions followed.
3.2.2. Purification of Neutrophils

Neutrophils are obtained from the bottom of each Ficoll–hypaque tube as follows:

1. Aspirate fluid above the erythrocyte/neutrophil pellet and add 5 mL of 0.2% saline.
2. Vortex for about 10 s.
3. Add 5 mL of 1.6% saline to restore isotonicity. This procedure lyses the erythrocytes and leaves the neutrophils intact.
4. Centrifuge 10 min at 246 g.
5. Remove supernatant and combine pellets in HBSS/HEPES in a 50-mL tube.
6. Wash the cells twice in HBSS/HEPES.
7. Resuspend the neutrophils in 10 mL of HBSS/HEPES and count on a hemocytometer.
8. Standardize to the desired concentration in the appropriate buffer for the assay.

3.3. Culture of Monocytes Into Mφ

3.3.1. Adherent Culture of Monocytes

1. Mixed MNCs are suspended to $4 \times 10^6$/mL in HBSS/HEPES containing 0.1% autologous serum.
2. One milliliter of cells is distributed to each well of a 24-well tissue culture plate containing a 13-mm diameter glass cover slip, and 0.1 mL is distributed to a 96-well plate, and the monocytes are allowed to adhere for 1 h at 37°C in 5% CO$_2$.
3. After 1 h, the lymphocytes are gently removed by swirling or gentle pipeting, and the monolayers washed twice in HBSS/HEPES.
4. The monocytes then are cultured in M199 at 37°C in 5% CO$_2$.
5. On day 3 or 4 the cells are given fresh media, and the Mφ are usually studied on day 7 (see Note 5[5]).

3.3.2. Suspension Culture of Monocytes

1. EasySep purified monocytes are suspended to $1 \times 10^6$/mL in RPMI-1640 containing 12.5% autologous serum and 10 µg/mL of gentamicin, and 10 mL of cells are dispensed into Teflon beakers.
2. The cells are harvested for study on days 5–7, and no intermediate feeding is required.

3.4. Culture of Monocytes Into DCs

1. EasySep purified monocytes are suspended to $6.5 \times 10^5$/mL in DC media, and 3 mL/well are dispensed into six-well tissue culture plates.
2. Add human recombinant granulocyte-macrophage colony-stimulating factor to a final concentration of 115 ng/mL and human recombinant interleukin-4 to a final
concentration of 50 ng/mL (Peprotech, Inc., Rocky Hill, NJ) and culture at 37°C in 5% CO₂.
3. The cells are harvested for study on days 5–8, and no intermediate feeding is required (3).

3.5. Binding Assay: General

Binding assays with Mφ and neutrophils are performed in Terasaki tissue culture plates (Miles Laboratories Inc., Naperville, IL). These plates are used as they hold a maximum volume of 15 µL. Thus, only a small number of cells, and small quantities of various reagents such as antibodies are required in this assay. Generally, the wells are coated with 1 mg/mL of human serum albumin for 1 h at RT. Alternatively, the wells may be coated with antibodies to down modulate specific receptors. After coating, the wells are washed twice with HBSA. This is done by flooding the plate with buffer and swirling vigorously. The HBSA then is poured off, and the remaining buffer in the wells is aspirated by touching the tip of a Pasteur pipet to the top edge of the well (5).

3.5.1. Macrophages

1. Five µL of Mφ standardized to 6 × 10⁵/mL in HBSA-AP (see Note 6) are added to each well and allowed to adhere for 1 h at 37°C in 5% CO₂.
2. The monolayers then are washed twice with HBSA. At this point, various reagents may be added to the wells depending on the experiment. These usually are added in 5 µL aliquots. The cells then are incubated at 4°C or 37°C for 30–60 min depending on the experiment. At the end of the preincubation period, these reagents may or may not be washed away as desired. Next, 5 µL of fluorescein isothiocyanate (FITC)-labeled HK Hc yeasts (1 × 10⁷/mL) are added to each well, and the plate incubated at 37°C in 5% CO₂ for 30 min.
3. Finally, the monolayers are washed twice with HBSA and the cells fixed overnight at 4°C in 1% paraformaldehyde.
4. The next day, the plates are rinsed in tap water, and the monolayers observed on an inverted microscope with phase and fluorescent optics.
5. Attachment of organisms is quantified by counting 100 Mφ per well and counting the total number of bound yeasts. The data are reported as the attachment index, which is the total number of yeasts bound per 100 Mφ.

3.5.2. Neutrophils

Neutrophils are standardized to 1 × 10⁶/mL in HBSA and 5 µL are added to each well and allowed to adhere for 1 h at 37°C in 5% CO₂. Experiments with neutrophils are performed following the same procedure as for Mφ.

3.5.3. Dendritic Cells

We have found that immature human DCs become adherent once they are removed from the presence of granulocyte-macrophage colony stimulating fac-
tor and interleukin-4. Further, at least for a period of 24 h, the DCs maintain their phenotype and do not differentiate into Mφ. Thus, one can perform the binding assay using adherent DC exactly as described above for Mφ. Alternatively, we have quantified attachment of Hc yeasts to DC in a suspension system.

1. DCs are harvested after 6–8 d of culture, then are washed and resuspended to $4 \times 10^6$/mL in HBSA. Fifty microliters of cells are dispensed into 12 × 75-mm polypropylene tubes.
2. At this point, various reagents such as monoclonal antibodies may be added, and the mixture incubated for 30 min at 4°C.
3. After preincubation with antibodies or other reagents, $1 \times 10^6$ FITC-labeled HK yeasts in 50 μL are added to each tube, and incubated for 30 min at 37°C in a water bath with orbital shaking at 150 rpm.
4. Ten microliters of sample is removed from each tube to a clean glass slide and cover slipped for immediate quantitation via phase and fluorescent microscopy.
5. One hundred DCs are counted per slide, and the results are expressed as the attachment index.

3.6. Phagocytosis Assay: General

Phagocytosis assays are performed with Mφ and neutrophils after adherence to 13-mm diameter glass cover slips in 24-well tissue culture plates. DCs also may be studied after adherence to glass cover slips as described in Subheading 3.5.3. for the binding assay. This has the advantage of being able to compare the properties of Mφ vs DC under identical conditions.

1. After adherence, the monolayers are washed twice with HBSA, and may be treated at this time with various reagents.
2. At the end of the treatment, the reagents may be left in or washed out.
3. One-milliliter aliquots of FITC-labeled yeasts (0.5–1 $\times 10^7$/mL) are added, and the mixtures incubated at 37°C for varying periods of time as desired.
4. At the end of the incubation period, nonadherent yeasts are removed from the monolayers by swirling the plates and washing twice with HBSA.
5. Trypan blue (0.3 mL of 1 mg/mL in PBS) is then added to each well for 15 min at 25°C to quench the fluorescence of bound but uningested yeasts.
6. The monolayers then are washed twice with HBSA and fixed overnight at 4°C in 0.5 mL of 1% paraformaldehyde.
7. After washing with distilled water, the cover slips are removed from the wells using a 21-gage needle with a bent tip and a pair of forceps.
8. The cover slips are mounted cell side down onto microscope slides in 5 μL of 90% glycerol in PBS and sealed with clear nail polish.
9. Phagocytosis is quantified via phase contrast and fluorescence microscopy at 1000×. One hundred cells are counted per cover slip, and the number of yeasts ingested (bright green), or bound but not ingested (dark green to red/brownish in color), are enumerated. Data can be presented as percent ingesting (the percent of
Mφ ingesting one or more yeasts); the phagocytic index (the total number of organisms ingested per 100 Mφ); and the association index (the total number of bound plus ingested organisms per 100 Mφ), or any combination of these as desired (5).

3.6.1. Macrophages

1. Mφ obtained by culture of monocytes on glass cover slips for 7 d can be used as is after washing twice with HBSA.
2. If suspension-cultured Mφ will be used, these are standardized to 2–3 × 10^5/mL in HBSA-AP.
3. One-milliliter aliquots of Mφ are added to the wells of a 24-well tissue culture plate containing glass cover slips and adhered for 1 h at 37°C in 5% CO₂.
4. The rest of the procedure is as described in Subheading 3.6.

3.6.2. Dendritic Cells

If DCs are to be studied adherently, they are standardized to 4 × 10^5/mL in DC media and 1-mL aliquots are added to the wells of a 24-well tissue culture plate containing glass cover slips, and the DCs allowed to adhere for 1 h at 37°C in 5% CO₂. The rest of the procedure is as described in Subheading 3.6. If the assay will be performed with DC in suspension, the procedure is as follows:

1. DCs are harvested from six-well plates after 6–8 d of culture, washed in HBSA, and standardized to 4 × 10^6/mL. 0.5 mL of DC are incubated with 0.5 mL of FITC-labeled HK Hc yeasts (2 × 10^7/mL) in a 17 × 100-mm polypropylene tube at 37°C in a water bath with orbital shaking at 150 rpm for varying periods of time.
2. Varying reagents may be added to the DC before the addition of the yeasts. At the end of the incubation period, 0.1 mL of Trypan blue (1 mg/mL in PBS) is added for 15 min at 25°C to quench the fluorescence of bound but uningested organisms.
3. One milliliter of HBSA is added to each tube, and the cells centrifuged at 200g for 5 min. Remove the top 1 mL, gently resuspend the cells, and then cytocentrifuge 50 µL of cell suspension onto a glass slide, and fix overnight in 1% paraformaldehyde at 4°C.
4. Rinse slides with distilled water and mount in 90% glycerol in PBS under a cover slip. Phagocytosis is quantified by phase-contrast and fluorescent microscopy as described in Subheading 3.6., step 9 (2).

3.6.3. Neutrophils

Neutrophils are standardized to 5 × 10^5/mL, and 1 mL aliquots are allowed to adhere to 13-mm diameter glass cover slips in 24-well tissue culture plates for 1 h at 37°C in 5% CO₂. The rest of the procedure is as described in Subheading 3.6.
3.7. Intracellular Growth Assay: General

This assay uses the uptake of $^3$H-leucine to quantify the number of yeasts remaining at the end of an experiment. Thus, the assay can be used to detect increased growth of yeasts and/or decreased growth of yeasts compared with a particular control. It should be noted that although 99% inhibition of growth may be obtained under certain experimental conditions, one still cannot make any determination as to the extent of killing of Hc yeasts. Thus, the assay is limited in this regard.

1. Cells adherent in 96-well tissue culture plates are treated with reagents such drugs or cytokines as desired. All treatments are done in triplicate, and treatments may be done just prior to infection with Hc yeasts, or for a period of 24–48 h or more before the addition of yeasts, such as when cytokines are used. The total volume of media and reagents is between 0.1 and 0.2 mL.

2. At the end of the treatment period, media is aspirated from the wells using a 27-gage needle (or left in as desired), and $1 \times 10^4$ viable yeasts in 0.1 mL of RPMI-1640 containing 5% heat-inactivated FCS and 10 $\mu$g/mL of gentamicin are added to each well.

3. After a further incubation for 24 h at 37°C in 5% CO$_2$, the plates are centrifuged at 384g for 10 min.

4. The supernatant is carefully aspirated through a 27-gage needle, and 50 $\mu$L of $^3$H-leucine (specific activity 153 Ci/mmol; New England Nuclear, Boston, MA) in sterile water (1 $\mu$Ci), and 5 $\mu$L of a 10X yeast nitrogen broth is added to each well.

5. After an additional incubation for 24 h at 37°C, 50 $\mu$L of L-leucine (10 mg/mL in water) and 50 $\mu$L of sodium hypochlorite (bleach) are added to each well.

6. The contents of the wells then are harvested onto glass fiber filters using an automated harvester (Skatron, Sterling, VA).

7. The filter discs are punched out into scintillation vials, 1 mL of Bio-Safe NA scintillation cocktail (Research Products International, Mount Prospect, IL) added, and the vials are counted in a Beckman LS6500 liquid scintillation spectrometer (Beckman Instruments Inc., Fullerton, CA) or equivalent (see Note 7 [6]).

3.7.1. Macrophages

1. Monocytes cultured into Mφ in 96-well tissue culture plates are used as is.

2. Suspension cultured Mφ are harvested and standardized to $6 \times 10^5$/mL in HBSA-AP.

3. One-tenth mL aliquots are dispensed into the wells of a 96-well tissue culture plate and allowed to adhere for 1 h at 37°C in 5% CO$_2$.

4. The buffer is aspirated and the experimental protocol is performed as described in Subheading 3.7.
3.7.2. Dendritic Cells

DCs are harvested and standardized to $6 \times 10^5$/mL in DC media and 0.1 mL aliquots are dispensed into the wells of a 96-well tissue culture plate. Other concentrations of DC may be utilized to change the ratio of DC:Hc. The DC may be allowed to adhere as desired. If adhered, then the media can be aspirated prior to the addition of reagents, drugs, and cytokines, etc. If left as is, the total volume of the assay still should not exceed 0.2 mL. Yeasts are then added and the protocol followed as described in Subheading 3.7. Because DCs inhibit the growth of yeasts, and yeasts replicate within Mφ, the intracellular growth of yeasts in Mφ are used as a positive control for intracellular growth. In addition, it may be desirable to culture the infected cells for 48 h to augment the difference between DC and Mφ. Alternatively, yeasts growing in media alone may be used as a positive control for the replication of yeasts (3).

3.7.3. Neutrophils

Neutrophils are standardized to varying concentrations in RPMI-1640 containing 10% normal human serum (to opsonize the yeasts) and 10 µg/mL of gentamicin depending on the desired ratio of neutrophils:Hc, and 0.1 mL dispensed into the wells of a 96-well tissue culture plate. At this point, various reagents, drugs, or cytokines can be added for varying periods of time prior to infection with Hc yeasts. Yeasts then are added and the protocol followed as described in Subheading 3.7. For experiments with neutrophils, yeasts in medium alone are included as a positive control for the replication of yeasts, because neutrophils inhibit the replication of yeasts (4).

4. Notes

1. For sonication of yeasts, the 50 mL conical tube is placed on ice and the yeasts sonicated for 30 s at a time at 40 W using a microprobe. The yeasts are allowed to cool down for one minute and the yeasts sonicated for another 30 s. This process is repeated until the yeasts are disrupted into mostly single yeasts and budding yeasts and must be determined empirically.
2. We do not use HK yeasts after 2 wk as they loose their “stickiness” for Mφ.
3. The concentration of FITC and the time of incubation must be determined empirically for a particular microscope. Although the concentration of FITC will not matter much for the binding assay, which is counted on an inverted microscope, the concentration of FITC used for labeling yeasts for the phagocytosis assay is critical. The yeasts must take up enough FITC so that they appear bright green within Mφ, but not so much that the fluorescence of yeasts outside the cell can not be quenched by trypan blue. For our Zeiss microscope, we label the yeasts with 0.01 mg/mL FITC for 15 min at RT.
4. The total amount of blood drawn and the number of tubes prepared will depend on the desired number of monocytes. For an average donor, one can expect to get approx $1 \times 10^6$ MNCs per mL of blood, of which approx 20–25% are monocytes.

5. Monocytes become “mature” Mφ after approx 5 d in culture. We usually study them after 7 d in culture for convenience. Thus, whatever day of the week the culture is started, the cells will be available for experimentation on the same day of the following week.

6. The aprotinin, which is a protease inhibitor, is added to the HBSA for the adherence of Mφ because the Mφ secrete some lysosomal enzymes during the adherence process. The aprotinin serves to neutralize these enzymes so they do not injure the Mφ.

7. As there is considerable variation in the cpm obtained from yeasts multiplying in untreated Mφ, neutrophils, or DCs from experiment to experiment, the data from several experiments usually is presented as the mean ± standard error of the mean of the percent inhibition of growth, which is defined as $1 - (\text{cpm in treated cells/ cpm in control cells}) \times 100$. This calculation also can give the percent increase in growth when applicable (6).

References


Immunology of Infection Caused by Cryptococcus neoformans

Floyd L. Wormley, Jr. and John R. Perfect

Summary

Cryptococcus neoformans is an opportunistic fungal pathogen that may lead to life-threatening meningoencephalitis and pulmonary infections in immunosuppressed hosts. The lack of an effective fungicidal regimen and the development of antifungal resistant strains suggest that continued investigation is necessary to devise immunotherapeutic strategies and/or drug targets to combat C. neoformans infections. Studies to date involve investigating the host–pathogen interaction of cryptococcal infections through the genetic manipulation of the yeast, as well as the characterization of the host immune response. Macrophage phagocytosis and killing assays have proven to be invaluable tools in evaluating the putative effects of the genetic manipulation of C. neoformans strains on the virulence composite of the yeast. In addition, the assay is used to assess the efficacy of various immunotherapeutic agents (i.e., antibodies and cytokines) to enhance this cell-based antifungal activity. The purpose of this chapter is to provide a brief overview on host immunity to C. neoformans infection and, in addition, describe a protocol for performing macrophage phagocytosis and killing assays with C. neoformans and its mutants.

Key Words: Cryptococcus neoformans, cryptococcosis; cryptococcus; meningitis; immunotherapy; nonspecific immunity; cell-mediated immunity; antifungal therapy; antibodies; phagocytosis, macrophages; J774 macrophage cell line.

1. Introduction

Cryptococcus neoformans, the etiological agent of cryptococcosis, is an encapsulated fungal pathogen that primarily infects the central nervous system of immunocompromised individuals, causing life-threatening meningoencephalitis (1). Although the yeast can cause disease in otherwise healthy individuals, it is largely an opportunistic pathogen for immunosuppressed hosts. C. neoformans is frequently found in soil with avian excreta, and infection is typically initiated via the inhalation of small (<5 µM in diameter) desiccated yeast.
cells or basidiospores. Deposition into the alveolar spaces will set up a pulmonary lymph node complex or pneumonitis, but the yeast shows a remarkable predilection to spread hematogenously into the CNS. Although the initial pulmonary infection is usually asymptomatic, those patients (70–90%) having clinical disease present with signs and symptoms associated with subacute meningitis or meningoencephalitis (headache, fever, lethargy, nausea/vomiting, coma, personality changes, and memory loss). The severity of disease and prognosis is largely dependent upon the integrity of host cellular defenses, inoculum size, and virulence characteristics of the yeast cells. Currently, there are numerous studies investigating the impact of each of these factors on the \textit{C. neoformans} disease process.

The initiation of effective host defenses against \textit{C. neoformans} is largely dependent on the ability of phagocytic cells—specifically, alveolar macrophages—to ingest and degrade the yeast cells and further enhance the inflammatory response. Studies are currently underway to characterize \textit{C. neoformans} virulence genes that mediate resistance to macrophage killing and growth inhibition, as well as the putative effects that various host defense mechanisms and immunotherapies have on augmenting macrophage effector cell responses. The use of macrophage phagocytosis and killing assays has proven to be an invaluable tool in characterizing \textit{C. neoformans}–macrophage interactions which is at the core of the host immune response to this encapsulated pathogenan (2–7). These assays are particularly useful given the recent developments of transformation systems and cloning techniques to evaluate potential \textit{C. neoformans} virulence genes. Studies on \textit{C. neoformans} virulence factors by site-directed mutants and specific effector cell functions are fundamental in evaluating the pathogenesis of cryptococcal infections and the assays discussed herein are important to investigate the intracellular pathogenesis of this yeast.

2. Materials

2.1. Maintenance of the Cell Line

1. J774.A.1, obtained from the American Type Culture Collection, is a murine (BALB/c) macrophage-like cell line derived from a reticulum cell sarcoma (see Note 1).
2. Dulbecco’s modified Eagle’s media (DMEM) culture media: DMEM supplemented with 10% heat-killed fetal calf serum (FCS; see Note 2), 1X nonessential amino acids, penicillin/streptomycin (100 µg/mL), and 10% NCTC-109 media.
3. 75-cm² cell culture flasks.
5. Cell culture is performed in a humidified incubator at 37°C with 5% CO₂ unless otherwise noted.
2.2. Isolation of Alveolar Macrophages

1. 70% Ethanol.
2. Surgical tools and equipment: surgical scissors and forceps (curved and straight) (keep in 70% ethanol), surgical silk thread, polyethylene tubing (PE50, Intramedic, Clay Adams™ Brand, Becton Dickinson, Sparks, MD), 25-gage needle, 50 mL of polypropylene conical centrifuge tubes, and a 1-mL tuberculin syringe.
3. 1X sterile phosphate buffered saline (PBS; pH 7.4).

2.3. Macrophage Phagocytosis/Killing Assay

1. For the activation of host alveolar macrophages or the J774A.1 cell line, DMEM culture media is supplemented with interferon-γ (100 µg/mL) and lipopolysaccharide (0.6 µg/mL).
2. Monoclonal antibody 18B7 (from Arturo Cassadevall, Albert Einstein College of Medicine, Bronx, New York).
3. Standard flat bottom, 96-well tissue culture plate.
4. 100% Ice-cold methanol.
5. Wright–Giemsa stain.
6. 0.5% Sodium dodecyl sulfate (SDS; see Note 3).
7. Yeast extract peptone glucose agar: combine yeast extract (10 g), peptone (20 g), glucose (20 g), agar (20 g), and 1000 mL of distilled water, mix well, and autoclave for 15 min at 121°C.

2.4. Nitric Oxide Assay

1. Griess Reagent (Sigma Aldrich, St. Louis, MO).
2. Standard 96-well microtiter plate.

3. Methods

3.1. Maintenance of Cell Lines

1. Cultures are propagated in DMEM complete media at 37°C with 5% CO₂ in 75-cm² cell culture flasks.
2. Carefully disassociate the cell monolayer from the bottom of the tissue culture flask using a cell scraper. Transfer the supernatant to a 50-mL conical tube and centrifuge for 10 min at 800g and 4°C.
3. Discard supernatant and resuspend cells in 10 mL of DMEM complete media.
4. Quantify viable cells using trypan blue dye exclusion and replate cells at a cell density of 1 to 2 × 10⁵ viable cells/mL.
5. Cells can be maintained by the addition of fresh medium or replacement of medium depending on cell density.
6. Cells can be stored in DMEM supplemented with 10% dimethylsulfoxide and 20% FCS.
3.2. Isolation of Alveolar Macrophages

1. Euthanize mice by CO₂ asphyxiation.
2. Wet the neck region of each mouse with 70% ethanol to sterilize the area.
3. Make a midline incision with sterile scissors and retract the skin with forceps.
4. Cut away fascia overlying trachea exposing the trachea (see Note 4).
5. Separate trachea from underlying tissues and esophagus using sterile curved forceps and place a 3-in. section of surgical silk thread underneath trachea.
6. Make a small incision in the upper quarter of the trachea and insert a 2-in. section of polyethylene tubing attached to a 25-gage needle on a 1-mL tuberculin syringe approx 3 mm into trachea and suture in place (see Notes 5 and 6).
7. Slowly inject 1 mL of sterile PBS through the tubing into the mouse lung, and collect the resultant bronchoalveolar lavage (BAL) and repeat until 10 mL has been collected.
8. Pool BAL fluid and centrifuge for 10 min at 800g.
9. Collect cell pellet, resuspend in DMEM media, and quantify cells using trypan blue dye exclusion.

3.3. Macrophage Phagocytosis/Killing Assay

Day 1:
1. Plate J774A.1 or primary alveolar cells at 1 × 10⁵ cells per well in a volume of 50 µL in a 96-well tissue culture plate.
2. Add 50 µL of DMEM culture media containing interferon-γ (100 µg/mL) and lipopolysaccharide (0.6 µg/mL) to those wells containing macrophages to be stimulated or 50 µL of DMEM culture media alone to non-stimulated macrophages and incubate the plate at 37°C with 5% CO₂ for 12–18 h.

Day 2:
1. Centrifuge an overnight culture of C. neoformans at 900g for 5 min, discard supernatant, and resuspend yeast pellet in 10 mL of sterile PBS (Repeat 3×).
2. After the final wash, resuspend the yeast cell suspension in 10 mL of DMEM culture media and quantify the number of viable yeast cells using trypan blue dye exclusion.
3. Resuspend yeast cell suspension to 1 × 10⁶ cells per mL in DMEM culture media containing mAb18B7 (1 µg/mL) and incubate for 1 h at 37°C with 5% CO₂ (see Note 7).
4. Add yeast cells (1 × 10⁵ cells/100 µL) to macrophages in 96-well tissue culture plate and incubate at 37°C at 5% CO₂ for 30 min to 1 h.
5. Wash wells (3×) with sterile PBS to remove extracellular yeast.

3.3.1. Determination of Phagocytic Index

1. Add 100 µL of 100% ice-cold methanol and incubate for 5 min at room temperature.
2. Remove methanol and add 50 µL of a 1:6 dilution of Wright–Giemsa stain and incubate for 5 min at room temperature.
3. Wash with sterile PBS (repeat 2×).
4. Quantify phagocytosis under oil-immersion microscopy (1000×), examining at least 100 cells. The phagocytic index (PI) is determined by the following formula:
   \[ PI = \frac{\text{number of attached and ingested cryptococci}}{\text{number of macrophages}}. \]

3.3.2. Phagocyte-Killing Assay

1. After the removal of extracellular yeast, add 200 µL of DMEM culture media to each well and incubate the plate at 37°C with 5% CO₂ for 24 to 48 h.
2. Remove DMEM culture media from wells and place into sterile 1.5-mL centrifuge tubes. To measure nitric oxide or cytokine levels, centrifuge or filter media through a 0.45 µm filter to remove the cells. Aliquot and store supernatant at −70°C for nitric oxide and/or cytokine measurement (see Note 8). Otherwise, proceed to step 3.
3. Add 100 µL of 0.5% SDS solution to each well and incubate at room temperature for 5 min.
4. Disrupt cells by aspirating and ejecting the lysate in each well with a pipet several times for complete cell disruption.
5. Add lysate to their respective 1.5-mL centrifuge tubes containing DMEM culture media.
6. Wash the wells a second time with 200 µL of sterile H₂O and add to previous tube.
7. Perform 1:10 dilution and spread 50 µL of each dilution on yeast extract peptone glucose agar.
8. Incubate plates at 30°C for 48–72 h and quantify phagocyte killing by counting colony-forming units.

3.4. Nitric Oxide Assay

1. Prepare serial dilutions of NaNO₂ solution (100–0 µM final concentrations) in DMEM complete media (see Note 9).
2. Pipet 50 µL of each standard and sample in duplicate or triplicate sets into wells of a 96-well microtiter plate.
3. Add 50 µL of Greiss reagent solution to NaNO₂ standards and samples and incubate at room temperature for approx 15 min.
4. Measure absorbance of NaNO₂ standards and samples at 540 nm using a microtiter plate reader. Calculate the mean absorbance values for each set of duplicate or triplicate standards and samples. Plot the standard curve and determine the µM amount of NO₂⁻ in each sample using the resulting standard curve.

3.5. Cytokine/Chemokine Assays

1. Cytokine quantification is usually accomplished using enzyme-linked immunosorbent assays, which are commercially available for the quantification of multiple cytokines/chemokines (BD Pharmingen, San Diego, CA; Genzyme, Boston, MA; Biosource International, Camarillo California, and R&D Systems, Minneapolis, MN).
4. Notes

1. Other cell lines that may be used in these phagocytosis and killing assays include the MH-S or RAW 264.7 macrophage cell lines. The protocols for performing the phagocytosis and killing assays using these cell lines are identical.

2. Inactivate complement proteins in FCS by incubating serum at 56°C for 30 min with intermittent mixing of bottle contents.

3. SDS causes skin and eye irritation. Therefore, eye protection, gloves, and a mask should be worn while dispensing SDS.

4. When dissecting away the fascia overlying the trachea, be careful not to sever the aorta.

5. Be very careful not to insert the tubing too far into the trachea. This will result in the dispensing of PBS into one lobe of the lungs.

6. Double knot the suture to secure the tubing in place. Do not remove tubing until BAL is complete to prevent leakage and loss of fluid.

7. Alternatively, opsonization of yeast cells by complement can be used to augment macrophage phagocytosis by incubating yeast cells in DMEM media containing fresh 10% mouse serum.

8. Multiple freeze/thaw cycles should be avoided to prevent denaturization of proteins.

9. Standards are prepared by diluting the stock NaNO₂ in the media being used (DMEM culture media). This will compensate for any nitric oxide that may be present in the media.

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With the growing frequency of fungal infections even as resistance to existing antifungal agents increases, methods for finding novel drug targets and evaluating antifungal resistance have become more important than ever. In Antifungal Agents: Methods and Protocols, expert scientists describe in detail the state-of-the-art molecular methods they have optimized for studying antifungal resistance, for discovering and evaluating both new and existing antifungal drugs, and for understanding the host response and immunotherapy of such agents. Many of the protocols were developed in the laboratory and, when a method is limited to specific organisms, include alternative techniques to expand their application. The protocols follow the successful Methods in Molecular Medicine™ series format, each offering step-by-step laboratory instructions, an introduction outlining the principle behind the technique, lists of the necessary equipment and reagents, and tips on troubleshooting and avoiding known pitfalls.

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