The role of antifungal susceptibility testing in the management of patients with invasive mycoses

Sofia Perea and Thomas F. Patterson

Department of Medicine, Div. Infectious Diseases, University of Texas, Health Science Center at San Antonio, Texas, USA

Summary

The availability of standardized antifungal susceptibility testing methodologies as well as the definition of interpretative breakpoints have made possible the establishment of useful correlations between *in vitro* testing data and clinical results with antifungal drugs such as fluconazole and itraconazole in patients with oropharyngeal candidiasis. The results obtained in these studies, however, can not be extrapolated to other organisms or clinical syndromes. Although there has been some recent progress, the interpretations of *in vitro* and *in vivo* results obtained in patients suffering cryptococcosis or invasive candidiasis needs to be further defined in order to establish meaningful clinical-laboratory correlations. Furthermore, the method needs to be fully standardized in case of filamentous fungi. It can be anticipated that the development, standardization and validation of *in vitro* antifungal susceptibility testing will guide clinicians in the management of patients with invasive mycoses.

Key words

Antifungal, Susceptibility testing, *Candida, Cryptococcus*, Filamentous fungi, Moulds

Papel de las pruebas de sensibilidad a los antifúngicos en el control de pacientes con infecciones fúngicas invasivas

Resumen

La disponibilidad de pruebas de sensibilidad a los antifúngicos estandarizadas así como la definición de puntos de corte ha hecho posible el establecimiento de la correlación entre los resultados obtenidos *in vitro* con la evolución clínica en el caso de antifúngicos tales como fluconazol e itraconazol en pacientes con candidiasis orofaríngea. Sin embargo, los resultados obtenidos en estos estudios no pueden ser extrapolados a otros hongos ni a otras situaciones clínicas. Aunque se han producido grandes avances, la interpretación de los resultados obtenidos *in vitro* e *in vivo* en pacientes con criptococosis o candidiasis invasiva debe ser estudiada en mayor profundidad para poder de ese modo establecer correlaciones entre los resultados clínicos y los resultados de laboratorio. En el caso de hongos filamentosos es necesario primero estandarizar y validar los métodos de sensibilidad a los antifúngicos. Se puede anticipar que el desarrollo, estandarización y validación de las pruebas *in vitro* de sensibilidad a los antifúngicos guiará a los clínicos en el control de los pacientes con infecciones fúngicas invasivas.

Palabras clave

Antifúngico, Pruebas de sensibilidad, *Candida*, *Cryptococcus*, Hongos filamentosos

Systemic mycoses are defined as fungal infections that invade beyond the superficial surfaces into tissues that are normally sterile. During the past two decades the frequency and types of life-threatening fungal infections have increased. Several factors have contributed to this rise: expansion of severely ill and/or immunocompromised patient population [human immunodeficiency virus (HIV) patients, cancer patients with chemotherapy-induced neutropenia, transplant recipients receiving immunosuppressive therapy, etc.]; the frequent use of more invasive medical procedures (extensive surgery, prosthetic devices and vascular catheters); treatment with broad-spectrum antibiotics or glucocorticoids; parenteral nutrition; and peritoneal dialysis or hemodialysis [1].

Dirección para correspondencia:
Dr. Thomas F. Patterson MD
Department of Medicine,
Div. of Infectious Diseases,
7703 Floyd Curl Drive, San Antonio,
Texas, 78284-7881, USA
Tel.: +1 210 567 6680; Fax: +1 210 567 3303
E-mail: patterson@uthscsa.edu.

1130-1406/99/5.00 Euros

Infections due to genera *Candida* and *Aspergillus* are important causes of morbidity and mortality in these population, representing 90% of all fungal infections [2]. In case of *Candida*, the rates of candidemia are increasing worldwide, representing the fourth most common nosocomial bloodstream infection in the United States [3,4]. Systemic candida infections are associated with a high mortality rate (38%), and a prolongation of hospital stay. Currently, *Candida albicans* accounts for approximately 50-60% of all nosocomial infections, although a noticeably shift in the species towards yeasts other than *C. albicans* (*Candida tropicalis*, *Candida krusei*, *Candida parapsilosis*, *Candida glabrata*) has occurred. Even though non-*C. albicans* species are considered less invasive and virulent than *C. albicans*, some species are inherently less susceptible to common antifungals which make them less amenable to treatment [5-8]. Mucosal *Candida* infection is a very common presentation in case of HIV patients. In HIV infected patients, other fungal infections such as cryptococcal meningitis, systemic endemic mycoses are also present, having an increased incidence with the progression of the HIV infection and reduction of the CD4 lymphocyte count [9,10]. In case of aspergillosis, although less frequent than candidiasis, it is associated with the highest mortality rate (27-77%) in severely immunocompromised populations such as patients undergoing allogenic bone marrow transplantation [11-14]. Several other less common fungi are becoming increasingly recognized as the source of deep fungal infections such as zygomycetes (*Rhizopus arrhizus*, *Absidia corymbifera*, and *Rhizomucor pusillus*), *Fusarium* spp., *Trichosporon beigelii*, *Blastoschizomyces capitatus*, and *Scedosporium* spp., *Acremonium* spp., *Malassezia furfur* and dematiaceous fungi [15-21].

This increase in the rate of opportunistic fungal infections has been accompanied by the development of new, less toxic and systemically active antifungal agents that represent therapeutic alternatives to amphotericin B such as fluconazole, itraconazole, the various amphotericin lipid formulations [amphotericin B lipid complex (Abelcet), amphotericin B colloidal dispersion (Amphocil), liposomal Amphotericin B (Ambisome)]; the new triazoles [voriconazole, SCH 56592, BMS-207147]; antifungals with new targets such as: echinocandines and pneumocandins (LY303366 and MK-0991); pradimicins (BMS 181184), and nikkomycin [22,23]. With the proliferation of antifungal agents, therapeutic options are more numerous, and the clinician must now select an agent which represents the best treatment strategy for a given patient. However, the growing number of reports on the development of drug resistance to one or more antifungal agents makes this decision more difficult [24-27].

In this review, we try to provide an overview of the development of standardized methodology for antifungal susceptibility testing, the establishment of interpretative breakpoints and its clinical application for the treatment of invasive mycoses.

In case of fungal infections, the ideal situation for clinicians would be to guide their antifungal therapeutic choice using the local epidemiological patterns of antifungal susceptibility testing. Certainly, the need to know whether a fungus causing invasive disease is susceptible or resistant to the antifungal selected is becoming increasingly important. But unlike antibacterial susceptibility testing, reliable antifungal susceptibility testing is still not commonly available in many laboratories. A decade ago, antifungal susceptibility testing was only occasionally performed and had not been carefully developed and standardized. This fact was translated into a very poor reproducibility and agreement of results obtained in intralaboratory and interlaboratory testing was not acceptable. In 1983, the National Committee for Clinical Laboratory for Clinical Laboratory Standards (NCCLS) responded to these problems by establishing a subcommittee to develop standardized antifungal susceptibility testing procedures that focused on broth-based methodologies with defined media. A number of investigators collaborated both independently and in cooperation with the Subcommittee to determine the role of different variables such as inoculum size, inoculum preparation, medium composition (liquid versus solid media), incubation time, temperature, volume, and endpoint definition in the standardization of the technique [28-33].

As a result of all these experiments, in 1997, the NCCLS approved the methodology for the standardization of broth-based macrodilution and microdilution methods for determination of the susceptibility of *Candida* species and *Cryptococcus neoformans* against amphotericin B, flucytosine, ketoconazole, itraconazole, and fluconazole [34]. This document, called M27-A and titled "Reference Method for Broth Dilution Antifungal Susceptibility Testing of Yeast" addressed the selection and preparation of antifungal agents, the implementation and interpretation of test procedures, as well as the quality control requirements for susceptibility testing of yeasts that cause invasive fungal infections (Table 1). Numerous studies using this methodology demonstrated interlaboratory reproducibility similar to that of antibacterial susceptibility testing [35,36].

Table 1. Summary of the M27-A methodology developed by the NCCLS for standardization of antifungal susceptibility testing for yeasts.

Factor	M27-A methodology
Methodology	Broth macrodilution, 1 ml final volume; or broth microdilution, 0.2 ml final volume
Medium	RPMI-1640 containing 0.165 M MOPS (Morpholinepropanesulfonic acid) pH, 7.0 [a]Antibiotic Medium 3 for Amphotericin B [b]Yeast Nitrogen Base for *C. neoformans* [c]Supplementation of test medium with glucose to a final concentration of 20g/l
Fungal inoculum	0.5-2.5 X 10^3 organisms
Incubation temperature	35°C
Incubation time	[d]48h (*Candida* species) or 72 h (*Cryptococcus neoformans*)
Endpoint	Amphotericin B; optically clear tube; [e]azoles and 5-flucytosine: 80% reduction in turbidity by comparison with growth control
Drugs and quality control (QC) isolates	Two QC isolates and corresponding QC ranges established via the M23 procedure are specified for amphotericin B, flucytosine, ketoconazole, itraconazole and fluconazole.

NOTE. Adapted from [55]
Some modifications were included for special circumstances:
[a] The use of Antibiotic Medium 3 may enhance detection of resistance, but this medium is not standardized and substantial lot-to-lot variability is possible [80,81].
[b] The use of Yeast Nitrogen Base may enhance the growth of *C. neoformans* and improve the clinical relevance of antifungal MICs [66].
[c] Supplementation of the test medium so that it contains glucose at a final concentration of 20 g/L may simplify endpoint determination [82].
[d] Improved interlaboratory reproducibility was observed when reading at 48h vs. 24h in case of amphotericin B, flucytosine, ketoconazole and fluconazole [83].
[e] This endpoint was used to handle the trailing growth phenomenon seen with the azole antifungal agents [33,84].

The next step was to correlate the clinical outcome with the *in vitro* results. Ideally, the results of *in vitro* antifungal susceptibility tests should provide a reliable prediction of *in vivo* response to therapy in human infections. However, the limitation of these highly artificial testing methods is such that only modest correlation exists between *in vitro* susceptibility testing and the outcome of the complex biological process that clinical infection

represents. Predicting clinical outcome is an extraordinary difficult issue, where the MIC is just a piece of the puzzle in which factors depending of the drug used, the host and the isolate itself participate (Table 2) [37-47]. The problem is, then, to determine the approximate relationship between the MIC and the likelihood of successful outcome, despite these interfering factors.

Table 2. Factors other than susceptibility *in vitro* influencing the clinical outcome of a fungal infection.

Factors	Variables
Drug pharmacokinetics	Dosing regimen, biodisponibility, drug stability, metabolism, drug interactions, protein binding, metabolites, tissue penetration, postantifungal effect.
Host factors	Patient compliance, immune system, type of infection, underlying disease
Site of infection	Source of infection, drug penetration, presence of foreign body (prosthetic devices, intravascular catheters), abscess formation,
Pathogen	Virulence factors; evasion of host inflammatory response, biofilm formation, decrease virulence by the acquisition of resistance

NOTE. Adapted from [55]

Prior to the establishment of interpretative breakpoints for antifungal susceptibility testing using the results obtained in clinical trials, attempts have been made to correlate the clinical outcome with *in vitro* results using animal models [48-53]. This method offers the advantage of fully integrating the effects of both the antimicrobial and the host factors. By performance of parallel studies of organisms that differ only in their susceptibility *in vitro*, the effect of MIC can be studied in detail. This technique is powerful, and studies with antifungal agents have often demonstrated general correlation with MIC and outcome. Unfortunately, animal models of infection may not necessarily mimic human infection. In addition, drug kinetics often differ substantially between man and other animals. However, these types of results can provide a very useful starting point for the establishment of the correlation between *in vitro* results and *in vivo* clinical outcome.

Candida

An early attempt to correlate *in vitro* antifungal susceptibility data with clinical outcome was published by Ghannoum *et al.* in 1996 [54]. In their review, they examined all the studies published in the literature that contained data about MIC and clinical outcomes in humans, and concluded that *in vitro* susceptibility testing could predict outcome only in selected clinical situations, such as fluconazole-treated AIDS patients with oropharyngeal candidiasis. In the case of more complex clinical situations, such as heterogeneous patients with invasive candidiasis, no such clear-cut correlation was present. One year later, tentative breakpoints for susceptibility testing of fluconazole and itraconazole MICs against *Candida* species were established largely using data from oropharyngeal candidiasis [55]. Four principles of interpretation of antimicrobial susceptibility testing were employed in the method: i) an MIC is not a physical or chemical measurement; ii) host factors are often more important than susceptibility test results in determining clinical outcome; iii) susceptibility *in vitro* may not always predict success of a particular therapy, but the possibility of failure for a particular drug or dosage when an infection is caused by a resistant isolate.

The data packages developed by the manufacturers of the antifungals fluconazole (Pfizer, USA) and itraconazole (Janssen Pharmaceutica, USA) that contained MICs of *Candida* isolates and outcome data from trials of therapy with either fluconazole and itraconazole for oropharyngeal candidiasis in patients with AIDS, and also, in the case of fluconazole, from patients with invasive candida infections were analyzed. In case of fluconazole, 636 *Candida* isolates from patients enrolled in six trials of fluconazole as therapy for oropharyngeal candidiasis in patients with AIDS (528 isolates: 77% *C. albicans*, 13% *C. glabrata*, 5% *C. tropicalis*, 3% *C. krusei*, and 2% other species) and from three trials of fluconazole as therapy for nonneutropenic patients with bloodstream and visceral candidiasis infection (108 isolates) were evaluated [56-62]. Based on the data analyzed, tentative breakpoints of ≤ 8 µg/ml as susceptible and ≥64 µg/ml as resistant were established. Isolates inhibited by fluconazole at concentrations of 16-32 µg/ml that respond to increased doses of fluconazole, were placed in the new category called susceptible dose dependent (S-DD). It was concluded that the response to fluconazole varied with the MIC, that is, higher doses of fluconazole can be used to treat patients infected with isolates for which MICs are higher; and failure of fluconazole therapy becomes likely when the MIC determined by NCCLS methodology exceeds the predicted peak serum levels of fluconazole expected for a given dosing regiment. These conclusions are strongest for patients with oropharyngeal candidiasis and *C. albicans* infection being more limited the available data for correlating MIC with outcome for non-*C. albicans* infections and for invasive candida infections. For *C. krusei*, the definition of susceptible and resistant does not apply since this organism is considered to be intrinsically resistant to fluconazole.

In case of itraconazole, 355 *Candida* species isolates (87% *C. albicans*, 9% *C. glabrata*, 2% *C. krusei* and 2% other species), from HIV patients enrolled in four trials of itraconazole solution as therapy for oropharyngeal candidiasis were studied [63]. As with fluconazole, consideration of the overall clinical data and their correlation with the pharmacokinetics of itraconazole allowed to conclude that the response of oropharyngeal candidiasis to itraconazole varies with MIC. Based on the data analyzed, tentative itraconazole breakpoints of ≤ 0.125 µg/ml as susceptible and ≥ 1.0µg/ml as resistant were established. Because infections due to isolates for which the itraconazole MIC are 0.25-0.5 µg/ml were observed to respond more often if higher itraconazole plasma levels were ensured, there were placed in the susceptible dose dependent category, that means susceptibility is dependant on achieving the maximal possible blood level.

These data were developed only in patients with mucosal infection, so that the extrapolation of these data to patients with invasive candidal infection is not established.

The guidelines for interpreting the MIC of fluconazole and itraconazole proposed represented a substantial advance in the process of making antifungal susceptibility a clinically useful tool (Table 3). Even though, it is important to remark on the limitations of the approach:

1. The breakpoint proposed are only valid for two drugs, fluconazole and itraconazole, and only for the *Candida* genus.
2. The *in vivo-in vitro* correlation for isolates at the higher MIC values obtained was not as strong in case of yeasts other than *C. albicans* and also in the case of systemic mycoses.

Table 3. Tentative breakpoints for fluconazole and itraconazole when the MIC is determined by M27-A[a,b].

Antifungal agent	Range of MICs (µg/ml) per category		
	Susceptible (S)	Susceptible-dose dependent(S-DD)[c]	Resistant (R)
Fluconazole	≤8	16-32	≥64
Itraconazole	≤0.125	0.25-0.5	≥1

[a] Isolates for *C. krusei* should be considered resistant to fluconazole regardless of the reported MIC to fluconazole
[b] Breakpoints values are applicable for MICs determined according to NCCLS-approved methods only
[c] Isolates having an MIC in the susceptible-dose dependent range should be treated with fluconazole 400-800 mg or an appropriate dose of itraconazole with results in serum concentration of ≥0.5µg/ml

Cryptococcus neoformans

C. neoformans is the cause of the most common life-threating fungal infection in patients with AIDS. Given the high incidence of relapse after initial antifungal therapy with amphotericin and flucytosine, the current management includes lifelong suppresive therapy fluconazole [64]. Chronic use of azoles for long-term suppressive therapy may become a factor for the selection of cryptococcal isolates that are more resistant to azoles, although the development of the fluconazole resistance has been very uncommon [65].

Throughout the development of the M27 methodology, it was clear that this approach was suboptimal for the testing of *C. neoformans*, because of the slow growth rate obtained in the RPMI 1640 broth, requiring 72 h of incubation, and because the fact that some strains did not even grow. A modification of the NCCLS method, using yeast nitrogen base medium buffered to a pH of 7.0, an inoculum of 10^4 cells/ml, and incubation at 35°C for 48 h in a microdilution format was developed. The MIC endpoint was read spectrophotometrically and for fluconazole was defined as 50% inhibition at 420 nm [66]. A multicenter evaluation of this method demonstrated an excellent level of interlaboratory agreement (96%) and an overall agreement of 90% with the M27 microdilution method [67]. There is only one study in which the correlation between the clinical outcome of patients with cryptococcal infection with the *in vitro* results for fluconazole was studied using this methodology, in which seventy-six patients with acute AIDS-associated cryptococcal meningitis that were treated with fluconazole ± flucytosine were enrolled. It was observed that those cases where the MIC of fluconazole was 0.25 µg/ml had a 25% treatment failure. This probability of failure increased to >80% for those whose MIC of fluconazole was 16µg/ml [68].

Filamentous fungi

The importance of susceptibility testing with pathogenic filamentous fungi has been less thoroughly studied compared to yeasts. Recently, a proposal method (M38-P) to standardize the *in vitro* antifungal susceptibility testing for moulds has been established (Table 4) [69]. Prior to this, studies to examine the role of different variables [inoculum size, type of inocula, incubation temperature, time of reading] as well as collaborative interlaboratory evaluations of the M27 reference method adapted to testing of moulds were developed. The initial collaborative six-center study evaluated the use of macrodilution and microdilution broth methods to determine the in vitro susceptibility of 25 isolates of filamentous fungi (*Aspergillus fumigatus*, *Aspergillus flavus*, *Pseudallescheria boydii*, *R. arrhizus*, and *Sporothrix schenckii*) to amphotericin B, fluconazole, itraconazole,

Table 4. Summary of the methodology developed for standardization of antifungal susceptibility testing for filamentous fungi (M38-P).

Factor	M38-P methodology
Methodology	Broth macrodilution, 1 ml final volume; or broth microdilution, 0.2 ml final volume
Medium	RPMI-1640 containing 0.165 M MOPS (Morpholinepropanesulfonic acid) pH, 7.0
Fungal inoculum	0.4-5 X 10^4 CFU/ml
Incubation temperature	35°C
Incubation time	21-26 h (*Rhizopus spp.*) 46-50 h (most other opportunistic filamentous fungi: *Fusarium* spp., *Aspergillus* spp., and *Sporothrix schenkii*) 70-74 h (*P. boydii*)
Endpoint	Amphotericin B; optically clear tube; azoles, flucytosine: ≥ 50% reduction in turbidity by comparison with growth control
Quality control (QC) isolates	*C. parapsilosis* ATCC 22019, *C. krusei* ATCC 6258, *A. flavus*[a] and *A. fumigatus*[a]

[a] ATCC numerical designation still pending

miconazole, and ketoconazole [70]. The results of this study were very encouraging and demonstrated excellent intralaboratory and interlaboratory agreement (90-100%) for macrodilution and microdilution methods in testing of amphotericin B, fluconazole, miconazole, and ketoconazole. A lower level of agreement (70-90%) was observed in the testing of itraconazole. A subsequent large-scale study involving 11 laboratories and 30 isolates representing six species of opportunistic mould pathogen showed a high level of interlaboratory agreement among the MICs determined by a broth microdilution adaptation of the M27 method [71]. The results obtained by other independent investigators using microdilution techniques following the M27 reference method with minor modifications (incubation temperature 25°C) were similar [72].

The clinical importance of mould infections in immunocompromised hosts cannot be overstated; however, the incidence of infections with most opportunistic mould pathogens is too low to permit a large-scale prospective comparison of antifungal MICs for moulds with the clinical results of antifungal treatment. For this reason, to date, there are minimal clinical data to support the relevance of filamentous fungi susceptibility testing *in vitro*. However, several studies in animal models have correlated efficacy with susceptibility results for some genera of moulds (Table 5) [73]. The results of the animal experiments carried out by Odds *et al.* in which the activities of amphotericin B and itraconazole were determined in relation of previously calculated MICs of the infecting isolates, for both drugs the treatment responses judged as showing some activity of the agent were associated with lower MICs than the responses considered as showing no activity *in vivo*. For the fungi for which the amphotericin B or the itraconazole MIC was less than 1 µg/ml, a response of some kind was seen in the experimental infections. For the fungi for which the amphotericin B MICs were at or above 2 µg/m or the itraconazole MICs were at or above 1µg/m, no response was seen. However, the overlap (amphotericin B) and 1-dilution difference (itraconazole) in MICs associated with response judged as active and inactive suggest that such MICs could not be interpreted as predicting treatment outcome in these animal models. The conclusion of the study was that just a limited association between MIC and treatment outcome was seen but that such association could be determined with confidence for less than half of the isolates studied because of the limitations of the animal model used.

Table 5. Evaluation of correlation between antifungal susceptibilities of filamentous fungi *in vitro* and antifungal treatment outcomes in animal infection models[a].

Results for amphotericin B			Results for itraconazole		
Fungus isolate (n)	MIC (µg/ml)	Response	Fungus isolate (n)	MIC (µg/ml)	Response
Rhizopus arrhizus (2)	0.25	Active	*Aspergillus fumigatus* (1)	0.25	Active
Aspergillus flavus (1)	1	Not active	*Aspergillus flavus* (1)	0.5	Active
Aspergillus fumigatus (1)	1	Active	*Pseudallescheria boydii* (2)	1	Not active
Fusarium solani (1)	1	Not active	*Rhizopus arrhizus* (2)	2	Not active
Fusarium oxysporium (2)	2	Not active/?[b]	*Fusarium oxysporium* (2)	>16	Not active/?[b]
Pseudallescheria boydii (2)	4	Not active	*Fusarium solani* (1)	>16	Not active

NOTE. Adapted from [73]
[a] The isolates are those used in reference [70] for the study of standardization of antifungal susceptibility testing.
[b] Results were too inconclusive for interpretation.

In case of endemic mycoses (*Coccidioides immitis*, *Histoplasma capsulatum*, *Blastomyces dermatitidis*, *Paracoccidioides brasiliensis*) very few studies have been made to determine the role of different variables such as inoculum size, type of inoculum, incubation temperature and time of reading in order to develop a reproducible method for antifungal susceptibility testing [74].

The field of antifungal susceptibility testing has progressed considerably since 1982. The development of standardized susceptibility testing procedures, quality control strains, and the optimization of the M27 method have placed antifungal susceptibility testing well within the reach of many clinical microbiology laboratories.

Currently, the recommended guidelines for studying clinical fungal isolates and antifungal susceptibility testing are as follows:

1. Periodic batch antifungal susceptibility testing of clinical isolates to establish the susceptibility for fluconazole and 5-FC against *Candida* spp.
2. Oropharyngeal candidiasis in patients with AIDS unresponsive to azole therapy (fluconazole, itraconazole, 5-FC)
3. Testing of isolates from deep sites, especially non-albicans isolates (fluconazole, itraconazole (rare), and flucytosine (rare).

In case of cryptococcal isolates, even though the results obtained with the broth microdilution method appear to be superior compared to those obtained with the NCCLS reference method, additional studies will be necessary to standardize this method and to allow its use in testing of *C. neoformans* against other antifungal agent. In case of mould infection, the routine testing for any class of isolates is still not recommended.

Evidence to support the clinical relevance of antifungal susceptibility testing will continue to grow as standardized methodology for yeasts and filamentous fungi evolves and large-scale surveys of clinical isolates are completed. Future efforts must be directed toward different issues such as:

1. Alternative approaches that are more convenient and easy to perform, in order to reduce the amount of work and the subjectivity and improve the results of current procedures, such as the E-test and disk diffusion techniques [75-76].
2. Improvement of the proposed methodology for filamentous fungi.
3. Establishment of interpretative breakpoints for the new antifungal agents under development, and for the currently available amphotericin B lipid formulations. Each of these new agents will pose additional challenges to the existing methodology, which may require additional adjustment in order to accurately reflect their clinically relevant antifungal activity [77].
4. Improvement of the correlation of *in vitro* results with *in vivo* clinical outcome in case of invasive *albicans* and no-*C. albicans* infections, as well as filamentous fungi invasive infections.

The currently high interest in fungal infections that has been stimulated by a raise in their incidence in immunocompromised patients has led to increased interest to antifungal susceptibility testing. Because of the long way we still have in front of us to determine a better correlation between clinical outcome in invasive fungal infections and MIC, the microbiology laboratory and clinicians must work to clarify the relative value of the antifungal susceptibility testing in the management of invasive mycoses, where a decision not to use a particular antifungal agent only because of a finding of "resistance" *in vitro* might have catastrophic consequences for the patient [78,79].

Work in the laboratory of TFP is supported by Public Health Service grants 1 R01 DE11381, and M01-RR-01346 for the Frederic C. Bartter General Clinical Research Center.

References

1. Lortholary O, Dupont B. Antifungal prophylaxis during neutropenia and immunodeficiency. Clin Microbiol Rev 1997;13: 477-504.
2. Warnock DW. Fungal infections in neutropenia: current problems and chemotherapeutic control. J Antimicrob Chemother 1998;41:95-105.
3. Pfaller MA, Jones RN, Doern GV, *et al.* International surveillance of blood stream infections due to *Candida* species from the SENTRY antimicrobial surveillance program in North America and Latin America, 1997 and 1998 [abstract no. F115, p. 319]. In program and abstract of the 99th General Meeting of the American Society for Microbiology, Chicago, IL, 1999.
4. Viscoli C, Girmenia C, Marinus A, Martino P, Meunier F. A surveillance study of fungemia in cancer patients in Europe. Invasive Fungal Infections Cooperative Group (IFIG of EORTC) [abstract 2]; Trends in Invasive Fungal Infections 3, Brussels, 1995.
5. Lewis RE, Klepser ME, Pfaller MA. Update on clinical antifungal susceptibility testing for *Candida* species. Pharmacotherapy 1998;18:509-515.
6. Wingard JR. Importance of *Candida* spe-

cies other than *C. albicans* as pathogens in oncology patients. Clin Infect Dis 1995; 20:115-125.
7. Pfaller MA. Nosocomial candidiasis: emerging species, reservoirs, and modes of transmision. Clin Infect Dis 1996;22 (Suppl 2): S89-S94.
8. Coleman DC, Rinaldi MG, Haynes KA, *et al.* Importance of *Candida* species other than *Candida albicans* as opportunistic pathogens. Med Mycol 1998;1:156-165.
9. Ampel NM. Emerging disease issues and fungal pathogens associated with HIV infection. Emerg Infect Dis 1996;2:109-116.
10. Wheat LJ. Diagnosis and management of fungal infections in AIDS. Curr Opin Infect Dis 1993;6:617-627.
11. Latgé JP. *Aspergillus fumigatus* and aspergillosis. Clin Microbiol Rev 1999;12:310-350.
12. Patel R, Paya CV. Infections on solid organ transplant recipients. Clin Microbiol Rev 1997;10:86-124.
13. Varthalitis I, Meunier F. Prophylaxis of fungal infections. In Meunier F (Ed.) Bailliere's clinical infectious diseases, vol. 2. Bailliere Tindall, London, 1995;157-177
14. Warnock DW. Fungal complications of transplantation: diagnosis, treatment and prevention. J Antimicrob Chemother 1995; 36:73-90.
15. Van den Saffele JK, Boelaert JR. Zygomycosis in HIV-positive patients: review of the literature. Mycoses 1996;39:77-84.
16. Annaissie E, Bodey GP, KantarjianH, *et al.* New spectrum of fungal infections in patients with cancer. Rev Infect Dis 1989;2:369-378.
17. Perfect JR, Schell WA. The new fungal opportunists are coming. Clin Infect Dis 1996;22(Suppl. 2):S112-S118.
18. Gamis AS, Gudnason T, Gieink GS. Disseminated infection with *Fusarium* in recipients of bone marrow transplants. Rev Infect Dis 1991;13:1077-1088.
19. Anaissie E, Kantarajan H, Ro J, *et al.* The emerging role of *Fusarium* infections in patients with cancer. Medicine 1988;67: 77-83.
20. Walsh TJ, Newman KR, Moody M, Wharon RC, Wade JC. Trichosporonosis in patients with neoplastic disease. Medicine (Baltimore) 1986;65:268-279.
21. Annaissie E. Opportunistic mycoses in the immunocompromised host: experience at a cancer center and review. Clin Infect Dis 1992;14(Suppl.1):S43-S53.
22. Georgepapadakou NH, Walsh TJ. Antifungal agents: chemotherapeutic targets and immunologic strategies. Antimicrob Agents Chemother 1996; 40: 279-291.
23. Sheehan DJ, Hitchcock CA, Sibley CM. Current and emerging azole antifungal agents. Clin Microbiol Rev 1999;12:40-79.
24. Rex JH, Rinaldi MG, Pfaller MA. Resistance of *Candida* species to fluconazole. Antimicrob Agents Chemother 1995;39:1-8.
25. Fichtenbaum CJ, Powderly WG. Refractory mucosal candidiasis in patients with human immunodeficiency virus infection. Clin Infect Dis 1998;26:556-565.
26. Franz R, Kelly SL, Lamb DC, Kelly DE, Ruhnke M, Morschhauser J. Multiple molecular mechanisms contribute to stepwise development of fluconazole resistance in clinical *Candida albicans* strains. Antimicrob Agents Chemother 1998;42: 3065-3072.
27. White TC, Marr KA, Bowden RA. Clinical, cellular, and molecular factors that contribute to antifungal drug resistance. Clin Microbiol Rev 1998;11:382-402.
28. Espinel-Ingroff A, Kerkering TM, Goldson PR, Shadomy S. Comparison study of broth macrodilution and microdilution antifungal susceptibility tests. J Clin Microbiol 1991; 29:1089-1094.
29. Guinet R, Nerson D, DeClosets F, *et al.* Collaborative evaluation in seven laboratories of a standardized micromethod for yeast susceptibility testing. J Clin Microbiol 1988; 26:2307-2312.
30. Shawar R, Paetnick V, Witte Z, Ensign LG, Anaissie E, LaRocco M. Collaborative investigation of broth microdilution and semisolid agar dilution for in vitro susceptibility testing of *Candida albicans*. J Clin Microbiol 1992;30:1976-1981.
31. Galgiani JN. Susceptibility testing of fungi: current status of the standardization progress. Antimicrob Agents Chemother 1993;37:2517-2521.
32. Pfaller MA, Rinaldi MG, Galgiani JN, *et al.* Collaborative investigation of variables in susceptibility testing of yeasts. Antimicrob Agents Chemother 1990;34:1648-1654.
33. Rex JH, Pfaller MA, Rinaldi MG, Polak N, Galgiani JN. Antifungal susceptibility testing. Clin Microbiol Rev 1993;6:367-381.
34. National Committee for Clinical and Laboratory Standards. Reference method for broth dilution antifungal susceptibility of yeasts. Approved standard M27-A. National Committee for Clinical and Laboratory Standards, Wayne PA, 1997.
35. Pfaller MA, Bale M, Buschelman B, *et al.* Quality control guidelines for National Committee for Clinical Laboratory Standards recommended broth macrodilution testing of amphotericin B, fluconazole, and flucytosine. J Clin Microbiol 1995;33: 1104-1107.
36. Rex JH, Pfaller MA, Lancaster M, Odds FC, Bolmstrom, Rinaldi MG. Quality control guidelines for National Committee for Clinical Laboratory Standards recommended broth macrodilution testing of ketoconazole and itraconazole. J Clin Microbiol 1996;34:816-817.
37. Pfaller MA, Rex JH, Rinaldi MG. Antifungal susceptibility testing: Technical advances and potential clinical applications. Clin Infect Dis 1997;24:776-784.
38. Drusano GL. Role of pharmacokinetics in the outcome of infections. Antimicrob Agents Chemother 1988;32:289-297.
39. Debruyne D, Ryckelnyck JP. Clinical pharmacokinetics of fluconazole. Clin Pharmacokinetic 1993;24.10-27.
40. Grant SM, Clissold SP. Itraconazole. Drugs 1989;37:310-344.
41. Barza M, Cuchural G. General principles of antibiotic tissue penetration. J Antimicrob Chemother 1985;15:59-75.
42. Wise R. The relevance of pharmacokinetics to *in-vitro* models: protein binding-does it matter? J Antimicrob Chemother 1985;15:77-83.
43. Hawser SP, Douglas LJ. Resistance of *Candida albicans* biofilms to antifungal agents. Antimicrob Agents Chemother 1995;39:2128-2131.
44. Rex JH. Catheters and candidemia. Clin Infect Dis 1996;22:467-70.
45. Minguez F, Chiu ML, Lima JE, Ñique R, Prieto J. Activity of fluconazole: postantifungal effect, effects of low concentrations and of pretreatment on the susceptibility of *Candida albicans* to leucocytes. J Antimicrob Chemother 1994;34:93-100.
46. Graybill JR, Montalbo E, Kirkpatrick WR, Luther MF, Revankar SG, Patterson TF. Fluconazole versus *Candida albicans*: a complex relationship. Antimicrob Agents Chemother 1998;42:2938-2942.
47. Sanders CC. ART versus ASTs: where are we going? J Antimicrob Chemother 1991; 28:621-625.
48. Anaissie EJ, Karyotakis NC, Hachem R, Dignani MC, Rex JH, Paetznick V. Correlation between *in vitro* and *in vivo* activity of antifungal agents against *Candida* species. J Infect Dis 1994;170: 384-389.
49. Van´t Wout JW, Mattie H, Van Futh R. Comparison of the efficacies of amphotericin B, fluconazole, and itraconazole against a systemic *Candida albicans* infection in normal and neutropenic mice. Antimicrob Agents Chemother 1989;33: 147-151.
50. Rogers TE, Galgiani JN. Activity of fluconazole (UK49,858) and ketoconazole against *Candida albicans in vitro* and *in vivo*. Antimicrob Agents Chemother 1986;30: 418-422.
51. Stiller RL, Bennett JE, Scholer HJ, Wall M, Polak A, Stevens DA. Correlation of *in vitro* susceptibility test results with *in vivo* response: flucytosine therapy in a systemic candidiasis model. J Infect Dis 1983;147: 1070-1077.
52. Fisher MA, Shen SH, Haddad J, Tarry WF. Comparison of *in vivo* activity of fluconazole with that of amphotericin B against *Candida tropicalis*, *Candida glabrata*, and *Candida krusei*. Antimicrob Agents Chemother 1989;33:1443-1446.
53. Barchiesi F, Najvar LK, Luther MF, Scalise G, Rinaldi MG, Graybill JR. Variation in fluconazole efficacy for *Candida albicans* strains sequentially isolated from oral cavities of patients with AIDS in an experimental murine candidiasis model. Antimicrob Agents Chemother 1996;40:1317-1320.
54. Ghannoum MA, Rex JH, Galgiani JN. Susceptibility testing of fungi: current status of correlation of *in vitro* data with clinical outcome. J Clin Microb 1996;34: 489-495.
55. Rex JH, Pfaller MA, Galgiani JN, *et al.* Development of interpretative breakpoints for antifungal susceptibility testing: conceptual framework and analysis of *in vitro-in vivo* correlation data for fluconazole, itraconazole, and candida infections. Clin Infect Dis 1997;24:235-247.
56. Quart AM, Espinel-Ingroff A, Steel- Moore L, Reich D. Evaluation of fluconazole in the treatment of oropharyngeal candidiasis in patients with AIDS. Infect Med 1996;13: 708-713.
57. Patterson TF, Revankar SG, Kirkpatrick WR, et al. Simple method for detecting fluconazole-resistant yeast with chromogenic agar. J Clin Microbiol 1996;34:1794-1797.
58. Espinel-Ingroff A, Quart A, Steel-More L, *et al.* Molecular karyotyping of multiple yeast species isolated from nine patients with AIDS during prolonged fluconazole therapy. J Med Vet Mycol 1996; 34: 111-116.
59. Sangeorzan JA, Bradley SF, He X, *et al.* Epidemiology of oral candidiasis in HIV-infected patients: colonization, infection, treatment, and emergence of fluconazole resistance. Am J Med 1994;97:339-346.
60. Webb ML, Gerberding JL, Hadley WK, Lee BL, Stansell JD, Sande MA. The prevalence of oral thrush and fluconazole resistant thrush in HIV+ patients [abstract no. 483] Clin Infect Dis 1995;21:800.
61. Redding S, Smith J, Farinacci G, *et al.* Resistance of *Candida albicans* to fluconazole during treatment of oropharingeal candidiasis in a patient with AIDS: documentation of *in vitro* susceptibility testing and DNA subtype analysis. Clin Infect Dis 1994;18:240-242.
62. Rex JH, Pfaller MA, Barry AL, Nelson PW, Webb CD. Antifungal susceptibility testing of isolates from a randomized, multicenter trial of fluconazole versus amphotericin B as treatment of nonneutropenic patients with candidemia. Antimicrob Agents Chemother 1995;39:40-44.
63. Graybill JR, Vazquez J, Darouiche RO, Morhart R, Moskovitz BL, Malego I. Itraconazole oral solution versus fluconazole treatment of oropharyngeal candidiasis [abstract no. I200] In program and abstract of the 35th Interscience Conference on Antimicrobial Agents and Chemotherapy, San Francisco, 1995.
64. Van der Host CM, Saag MS, Cloud GA, *et al.* Treatment of cryptococcal meningitis associated with the acquired immunodeficiency syndrome. N Engl J Med 1997; 337: 15-21.
65. Currie BP, Ghannoum M, Bessen L, Casadevall A. Decreased fluconazole susceptibility of a relapse *Cryptococcus neoformans* isolate after fluconazole treatment. Infect Dis Clin Pract 1995;4:318-319.
66. Ghannoum MA, Ibrahim AS, Fu Y, Shafiq MC, Edwards JE, Criddle RS. Susceptibility testing of *Cryptococcus neoformans*: a microdilution technique. J Clin Microbiol 1992;30:2881-2886.
67. Sanati H, Messer SA, Pfaller M, *et al.* Multicenter evaluation of broth microdilution method for susceptibility testing of *Cryptococcus neoformans* against fluconazole. J Clin Microbiol 1996;34:1280-1282.
68. Witt MD, Lewis RJ, Larsen RA, *et al.* Identification of patients with acute AIDS-associated cryptococcal meningitis who can be effectively treated with fluconazole: the role of antifungal susceptibility testing. Clin Infect Dis 1996;22:322-328.
69. National Committee for Clinical and Laboratory Standards. Reference method for broth dilution antifungal susceptibility of conidium-forming filamentous fungi; Proposed standard. NCCLS document M38-P. Wayne, PA, 1998.

70. Espinel-Ingroff A, Dawson K, Pfaller M, *et al.* Comparative and collaborative evaluation of standarization of antifungal susceptibility testing for filamentous fungi. Antimicrob Agents Chemother 1995;39: 314-319.
71. Espinel-Ingroff A, Barlett M, Bowden R, *et al.* Multicenter evaluation of proposed standarized procedure for antifungal susceptibility testing of filamentous fungi. J Clin Microbiol;1997;35:139-143.
72. Pujol I, Guarro J, Llop C, Soler L, Fernandez-Ballart J. Comparison study of broth macrodilution and microdilution antifungal susceptibility tests for the filamentous fungi. Antimicrob Agents Chemother 1996;40:2106-2110.
73. Odds FC, Van Gerven F, Espinel-Ingroff A, *et al.* Evaluation of possible correlations between antifungal susceptibilities of filamentous fungi *in vitro* and antifungal treatment outcomes in animal infection models. Antimicrob Agents Chemother 1998;42:282-288.
74. Gonzalez G, Tijerina R, Sutton DA, Rinaldi MG. Standarized inoculum preparation studies for the antifungal susceptibility testing of the saprobic phase of *Coccidioides immitis*, abstr C-172, p.139. In Program and abstracts of the 99th General Meeting of the American Society for Microbiology, Chicago, IL, 1999.
75. Espinel-Ingroff A, Pfaller M, Messer SA, *et al.* Multicenter comparison of the sensitre YeastOne colorimetric antifungal panel with the National Committee for Clinical Laboratory Standards M27-A reference method for testing clinical isolates of common and emerging *Candida* spp., *Cryptococcus* spp., and other yeasts and yeast-like organisms. J Clin Microbiol 1999;37:591-595.
76. Clancy CJ, Nguyen MH. Correlation between *in vitro* susceptibility determined by E test and response to therapy with amphotericin B: results from a multicenter prospective study of candidemia. Antimicrob Agents Chemother 1999;43: 1289-1290.
77. Oakley KL, More CB, Denning DW. Comparison of *in vitro* activity of liposomal nystatin against *Aspergillus* species with those of nystatin, amphotericin B desoxycholate, AB colloidal dispersion, liposomal AB lipid complex, and itraconazole. Antimicrob Agents Chemother 1999;43: 1264-1266.
78. Verbist L. Relevance of antibiotic susceptibility testing for clinical practice. Eur J Clin Microbiol Infect Dis 1993;12:2-5.
79. Odds FC. Personal opinion: can antifungal sensitivity tests predict clinical treatment outcomes? Rev Iberoam Micol 1997;14:83-84.
80. Rex JH, Cooper CR, Mertz WG, Galgiani JN, Anaisse EJ. Detection of amphotericin B-resistant *Candida* isolates in a broth-based system. Antimicrob Agents Chemother 1995;39:906-909.
81. Lozano-Chiu M, Nelson PW, Lancaster M, Pfaller MA, Rex JH. Lot-to-lot variability of antibiotic medium 3 used for testing susceptibility of *Candida* isolates to amphotericin B. Antimicrob Agents Chemother 1997; 35:270-272.
82. Rodriguez-Tudela JL, Martinez–Suarez JV. Improved medium for fluconazole susceptibility testing of *Candida albicans*. Antimicrob Agents Chemother 1994;38:45-48.
83. Revankar SJ, Kirkpatrick WR, McAtee RK, *et al.* Interpretation of trailing endpoints in antifungal susceptibility testing by the National Committee for Clinical Laboratory Standards Method. J Clin Microbiol 1998;36:153-156.
84. Rex JH, Nelson PW, Paetznick VL, Lozano-Chiu M, Espinel-Ingroff A, Anaissie EJ. Optimizing the correlation between results of testing *in vitro* and therapeutic outcome *in vivo* for fluconazole by testing critical isolates in a murine model of invasive candidiasis. Antimicrob Agents Chemother 1998;42:129-134.