SUSCEPTIBILITY TEST FOR FUNGI: CLINICAL AND LABORATORIAL CORRELATIONS IN MEDICAL MYCOLOGY

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SUMMARY

During recent decades, antifungal susceptibility testing has become standardized and nowadays has the same role of the antibacterial susceptibility testing in microbiology laboratories. American and European standards have been developed, as well as equivalent commercial systems which are more appropriate for clinical laboratories. The detection of resistant strains by means of these systems has allowed the study and understanding of the molecular basis and the mechanisms of resistance of fungal species to antifungal agents. In addition, many studies on the correlation of in vitro results with the outcome of patients have been performed, reaching the conclusion that infections caused by resistant strains have worse outcome than those caused by susceptible fungal isolates. These studies have allowed the development of interpretative breakpoints for Candida spp. and Aspergillus spp., the most frequent agents of fungal infections in the world. In summary, antifungal susceptibility tests have become essential tools to guide the treatment of fungal diseases, to know the local and global disease epidemiology, and to identify resistance to antifungals.

KEYWORDS: Susceptibility test; Fungi.

INTRODUCTION

Fungal infections are a major cause of morbidity and mortality despite the latest developments of diagnostic tools and therapeutic options. Early initiation of the correct antifungal therapy has been demonstrated to have a direct impact on the patient’s outcome. More severe infections affect mainly immunocompromised patients but other populations are also infected. New chronic lung infections have been described with a huge impact on the patient’s quality of life, and a high cost of treatment and care. Besides, some skin fungal infections involving mucosa and subcutaneous tissues cause substantial morbidity. Cryptococcus, Candida, Aspergillus, and Pneumocystis are the main etiologic agents of fungal infections. The burden and mortality associated with these diseases depend on the region and the affected population. Thus, it has been estimated that cryptococcal meningitis affects nearly one million people per year. Despite treatment, mortality rates can reach 55 to 70% in AIDS patients in Latin America and sub-Saharan Africa, the estimated number of deaths per year being over 620,000.

Cryptococcus, Candida, Aspergillus, and Pneumocystis affect mainly immunocompromised individuals, however endemic dimorphic fungi such as Histoplasma, Blastomyces, Coccidioides and Paracoccidioides affect immunocompetent patients as well, and endemic areas include several regions of Latin America.

Nowadays, three main families of antifungals are used in the clinical setting to treat fungal infections: polyenes represented by amphotericin B (and its different formulations); azoles with several derivatives such as itraconazole, fluconazole, voriconazole, posaconazole, isavuconazole; and the echinocandins caspofungin, micafungin and anidulafungin. The availability of new antifungals in recent years has provided clinicians with more options, increasing the use of these compounds not just for treatment when the infection has been diagnosed, but also as prophylactic, empirical or preemptive treatment. The increased use of antifungals has induced a higher selective pressure on fungal strains and resistance has emerged in two main ways: several species have developed secondary resistance and susceptible species have been replaced by resistant ones, changing the epidemiology of fungal infections.

Antifungal susceptibility testing methods are available to detect antifungal resistance and to determine the best treatment for a specific fungus. Clinical microbiology relies on these methods to select the agent of choice for a fungal infection, and to know the local and the global epidemiology of antifungal resistance.

Microdilution methods are the gold standard or reference techniques. Two organizations, the European Committee on Antibiotic Susceptibility Testing (EUCAST) and the Clinical Laboratory Standards Institute (CLSI) have standardized methods to perform antifungal susceptibility testing. Differences between these two methods have been widely...
discussed in several reports and will be reviewed in the present manuscript, however their results have demonstrated to be comparable and are used worldwide. Both institutions have developed breakpoints (BPs) of some antifungals to *Candida* and *Aspergillus* species that are currently used to classify resistant strains.

Regardless of their advantages, the standardized broth microdilution methods of antifungal susceptibility testing are time-consuming and cumbersome for clinical laboratories. Some commercially available, including manual, semi-automated and automated methods, do not require complex handling and are cost-effective alternative methods to test antifungal agents in *vitro* against *Candida* isolates in routine usage, and *Cryptococcus* isolates and filamentous fungi for research purposes. The characteristics of these methods together with their comparison with the reference procedures and the available agar-based methods will also be reviewed in this manuscript.

**REFERENCE METHODS**

**Broth microdilution methods**

Clinical Laboratory Standards Institute (CLSI)

In 1985, the CLSI, formerly known as the National Committee for Clinical Laboratory Standards (NCCLS), formed a subcommittee on Antifungal Susceptibility Testing that published, in 1997, the document M27A “Reference Method for Broth Dilution Antifungal Susceptibility Testing of Yeast; Approved Standard”\(^9\). This document defined reference strains with ranges of Minimal Inhibitory Concentrations (MIC) and Break Points (BPs) for some antifungals and their action against yeasts such as *Candida* and *Cryptococcus*. Since then, several updates have been published, the current one having been approved in April 2008\(^20\). For filamentous fungi, the first document was published in 2002: M38A: “Reference Method for Broth Dilution Antifungal Susceptibility Testing of Filamentous Fungi; Approved Standard” with a second edition published in 2008, which is the currently accepted one\(^21\).

European Committee on Antimicrobial Susceptibility Testing (EUCAST)

The EUCAST is a standing committee jointly organized by the European Society of Clinical Microbiology and Infectious Diseases (ESCMID), the European Centre for Diseases Control (ECDC), and the European National Breakpoint Committees (www.eucast.org). The antifungal susceptibility testing subcommittee of the EUCAST (AFST-EUCAST) was formed in 1997, and in 2008 published a standard of susceptibility testing for yeasts (including *Cryptococcus*). This standard was updated in 2012\(^9\). Another standard for molds was published in 2008. All of these standards are available online and can be downloaded from the EUCAST website (www.eucast.org). Differences between both standards are mainly found in the inoculum size, incubation time and medium composition (Table 1). Despite these differences, the results obtained by both methods are comparable\(^15,55\). CLSI and EUCAST include in their standards to test yeast some modifications for *Cryptococcus*. Thus, in CLSI the recommendation is to read MICs for *Cryptococcus* after 70 to 74 hours of incubation (in contrast with 24-48h for *Candida*), while EUCAST recommends the incubation of the plates at 30 °C when the growth control does not reach an optical density of 0.2 at 35 °C. Neither CLSI nor EUCAST have published standards for endemic dimorphic fungi such as *Histoplasma* or *Paracoccidioides*.

| Well shape (bottom) | Media | Glucose content | Inoculum size | Incubation temperature | Incubation time | Reading | Endpoint |
|---------------------|-------|----------------|----------------|------------------------|----------------|---------|----------|
| round               | RPMI 1640 | 0.2%       | 0.5-2.5 x 10\(^6\) | 35 °C                 | 24h             | Visual  | AMB, FCZ, Candins |
| flat                | RPMI 1640 | 2%         | 0.5-2.5 x 10\(^6\) | 35 °C                 | 24h             | Spectrophotometric | Azoles and Candins |

Differences between two methods are in bold. AMB = amphotericin B; FCZ = fluconazole; Candins = anidulafungin, caspofungin, micafungin.

**Agar-based methods**

Disk tests are inexpensive and easy to set up, and provide an ideal screening test. The disk diffusion method to test antifungals (CLSI M44 series) has been developed and validated only in the case of azoles and echinocandins for *Candida* spp. isolates\(^16\). It recommends the use of Mueller-Hinton agar supplemented with 2% glucose, providing a suitable growth for most yeasts, and 0.5 mg/L methylene blue dye medium (enhances the zone edge definition) minimizing the trailing effect. The pH of the medium needs to be between 7.2 and 7.4 after gelling and the agar should be 4 cm high. The inoculum is standardized to 0.5 McFarland using a densitometer and plates should be incubated at 35 °C for 24 h; some strains show insufficient growth and may need 48 h of incubation. In addition, quality control parameters have been established following the CLSI standard procedures. The results of the susceptibility test according to the zone diameter interpretative criteria for caspofungin, fluconazole and voriconazole for *Candida* species allows to classify the isolate in one of the following categories: susceptible, resistant, susceptible dose dependent and non-susceptible, corresponding to MIC breakpoints\(^23\).

The essential agreement between the disk diffusion and the CLSI microdilution method to test the susceptibility of azoles against *Candida* and *Cryptococcus* isolates is usually higher than 90% demonstrating that the disk diffusion is able to identify resistant isolates\(^11,35\). Regarding echinocandins and *Candida* species, the disk diffusion test appears to be able to differentiate caspofungin-susceptible between resistant mutant isolates. However, the disk diffusion test for micafungin appears to be less optimal due to a close overlapping of susceptible and resistant
populations. In the case of *C. parapsilosis* and *C. glabrata*, there is a need for individual breakpoints. This behavior has been observed with either EUCAST or CLSI microdilution methods and thus appears to be drug-related rather than dependent on the choice of the *in vitro* susceptibility test format. Nevertheless, while susceptibility classification is improved by the application of recently revised breakpoints, further evaluation and refinement are needed.

The standard disk diffusion method to test antifungal drugs for non-dermatophyte filamentous fungi isolates (M51-A and supplement M51-S1) provides qualitative results in 8-24 h when caspofungin, triazoles, and amphotericin B are used, faster than the CLSI reference microdilution method. Among *Aspergillus* species, a lower agreement of results produced by disk diffusion susceptibility tests was reported for *A. flavus* and amphotericin B or voriconazole. Amphotericin B to test *A. fumigatus* susceptibility also showed a lower agreement when compared to the reference method. Amphotericin B disks usually show the lowest correlation between MICs and inhibition zone diameters for filamentous fungi. The percentage of major errors is usually similar to that obtained with the itraconazole disk, but the percentage of minor errors is higher. Although breakpoints for filamentous fungi have not been defined, epidemiological cut-off values can be proposed to identify non-wild-type isolates.

Although qualitative results provided by the disk diffusion method are useful in the clinical laboratory routine, quantitative MIC data is somewhat critical for the management of invasive infections.

### Breakpoints

Even though the main goal of AFST is to select the best treatment for a given isolate, these methods are also very important to detect resistant strains, allowing the establishment of an epidemiology map of antifungals resistance that is an emerging problem in medical mycology. The two main factors are: the development of secondary resistance and the selection of species that are intrinsically resistant. Therefore, AFST has become critical for the choice of the best antifungal agent. Breakpoints have been developed for some fungal species and antifungals, in both CLSI and EUCAST methods. These BPs categorize fungal isolates into (i) susceptible (the drug is an appropriate treatment); (ii) resistant (the drug is not recommended as a treatment), and (iii) intermediate (the drug may be an appropriate treatment, depending on certain conditions; e.g. fluconazole to treat a urinary infection caused by an intermediate strain).

BP definition is a complex process based on the critical review of several aspects and data. CLSI evaluates MIC distributions, the relationship between MICS and clinical outcome, pharmacokinetics and pharmacodynamics. CLSI proposed a single interpretative BPs for fluconazole, itraconazole, voriconazole, and echinocandins for all *Candida* species. Latter, CLSI BPs was revised including a number of clinical studies and cases reporting strains classified as susceptible but associated with treatment failure, and as a consequence, species-specific BPs were proposed, as had been previously established by EUCAST.

EUCAST evaluates five aspects to develop BPs: (i) The most common dosage used in each European country; (ii) the definition of the wild type population for each target microorganism at the species level, and the determination of epidemiological cut-offs; (iii) the pharmacokinetics of the drug; (iv) the pharmacodynamics including Monte Carlo simulations; and (v) the correlation of MICs with patients’ clinical outcome treated with this drug. Clinical BPs have been established for several antifungals for *Candida* spp. and *Aspergillus* spp. These BPs are freely available online at: www.eucast.org/clinical_breakpoints.

As stated before, although some differences have been recognized for several years, currently CLSI and EUCAST breakpoints are in agreement. Tables 2 and 3 represent the established BPs for several antifungals for *Candida* and *Aspergillus* in both standards.

### Resistance

Antifungal resistance is becoming an emerging problem. On the one hand, there is the intrinsic resistance, and on the other hand the development of secondary resistance, that should be detected because resistant strains are associated with poorer outcomes. To illustrate this problem, intrinsic resistance of *C. glabrata* and *C. krusei* to fluconazole is well known. In these cases, appropriate treatment can be decided on the basis of species identification. This intrinsic resistance has justified the use of echinocandins as primary treatment, instead of fluconazole, in the empirical treatment of candidemia and invasive candidiasis in recently published guidelines. In addition, intrinsic resistance to echinocandins has been described in *C. parapsilosis*, and *Cryptococcus neoformans* isolates.

Although it is less common, during antifungal therapy acquired resistance in *Candida* spp. infections has also been reported. Most cases involve *C. glabrata* resistance to echinocandin although other species such as *C. albicans*, *C. tropicalis* and *C. krusei*, have also proven able of developing secondary resistance. Alterations on genes encoding the target enzymes of these drugs (beta 1-3 D-glucan synthase for echinocandins (FKS) and 14 alpha sterol demethylase for azoles (ERG11) or up regulation of multidrug efflux transporters also for azoles (ABC [ATP-binding cassette]/MFS [major facilitator superfamily]) have been blamed for the *Candida* spp. resistance to antifungal agent. Point mutations located at two hot spot regions within the FKS genes of *Candida* spp. have been described and associated with echinocandins resistance.

Secondary resistance to amphotericin B has been described in *C. tropicalis*, *C. parapsilosis*, *C. lusitaniae*, and *C. haemulonii*. In addition, several emerging pathogens such as *A. terreus*, *Fusarium* spp, and *Lomentospora prolificans* (syn. *Scedosporium prolificans*) are intrinsically resistant to amphotericin B. The mechanism of resistance to amphotericin B has been associated with a decrease of ergosterol content in fungal membranes, mainly due to alterations in the ergosterol biosynthesis pathway. It has also been suggested that resistance to amphotericin B could be related to disruption of the fungal mitochondria.

The azole resistance of *Aspergillus* isolates has been rigorously investigated in the last years. Alterations in the coding region of the cyp51A gene (positions G54, G138, M220, G448) or an insertion of a 34 to 36 base pair tandem repeat in the promoter region of the gene, together with point mutations (positions L98, Y121 and T289) have been associated with azole resistance. Mechanisms of azole resistance have been described both prior to triazole exposure and acquired during therapy. The use of azoles in agriculture has been described as a cause of the emergence of triazole resistant in *Aspergillus fumigatus* isolates, particularly in Europe and Asia.
Other filamentous fungi are intrinsically resistant to some antifungals. The order mucorales comprises several pathogenic species that are resistant to voriconazole\(^1\). Species of the genera *Fusarium* and *Scedosporium* also show elevated MICs of several antifungals\(^3,26\). In addition, multi-resistant species are also present as human pathogens. *Lomentospora prolificans* (syn. *Scedosporium prolificans*) is resistant to all azoles, echinocandins and amphotericin B, and has been associated with poorer outcomes\(^54\).

### Commercial methods

Clinical laboratories can determine susceptibility to antifungals through a series of commercially available systems, including the Sensititre YeastOne® panel (TREK Diagnostic Systems, Cleveland, USA) and the Vitek 2 system, both based on microdilution methods, or agar-based assays, e.g. test strips (E-Test®, bioMérieux; MIC®, Oxoid) and discs impregnated with a single antifungal agent.

In order to choose a commercial method, first of all, the laboratory should be aware of the commercial techniques results considering the susceptibility of each drug to a particular fungus, comparing the CLSI and the EUCAST reference procedures. In general, the correlation is based on the essential agreement (EA), defined as the discrepancies between MIC results of no more than ± 2 twofold dilutions, and the categorical agreement (CA) defined as the percentage of results that are within ± 1 twofold dilution of each other.

### Table 2

**EUCAST and CLSI antifungal breakpoints for *Candida***

| Antifungal agent | Standard | *C. albicans* | *C. glabrata* | *C. krusei* | *C. parapsilosis* | *C. tropicalis* |
|------------------|----------|---------------|---------------|-------------|------------------|-----------------|
| AMB              | EUCAST   | S ≤ | R > | S ≤ | R > | S ≤ | R > | S ≤ | R > | S ≤ | R > | S ≤ | R > |
|                  | CLSI     | -   | -   | -   | -   | -   | -   | -   | -   | -   | -   | -   |   |
| ANF              | EUCAST   | 0.03 | 0.03 | 0.06 | 0.06 | 0.06 | 0.06 | 0.002 | 4 | 0.06 | 0.06 |
|                  | CLSI     | 0.25 | 0.5 | 0.12 | 0.25 | 0.25 | 0.5 | 2 | 4 | 0.25 | 0.5 |
| CPF              | EUCAST   | -   | -   | -   | -   | -   | -   | -   | - |
|                  | CLSI     | 0.25 | 0.5 | 0.125 | 0.25 | 0.25 | 0.5 | 2 | 4 | 0.25 | 0.5 |
| FCZ              | EUCAST   | 2 | 4 | 0.002 | 32 | - | - | 2 | 4 | 2 | 4 |
|                  | CLSI     | 2 | 4 | 32 (SDD) | 32 | - | - | 2 | 4 | 2 | 4 |
| ICZ              | EUCAST   | 0.06 | 0.06 | IE | IE | IE | IE | 0.12 | 0.12 | 0.12 | 0.12 |
|                  | CLSI     | 0.125 | 0.5 | 0.125 | 0.5 | 0.125 | 0.5 | 0.125 | 0.5 | 0.125 | 0.5 |
| MCF              | EUCAST   | 0.016 | 0.016 | 0.03 | 0.03 | IE | IE | 0.002 | 2 | IE | IE |
|                  | CLSI     | 0.25 | 0.5 | 0.06 | 0.12 | 0.25 | 0.5 | 2 | 4 | 0.25 | 0.5 |
| PCZ              | EUCAST   | 0.06 | 0.06 | IE | IE | IE | IE | 0.06 | 0.06 | 0.06 | 0.06 |
|                  | CLSI     | - | - | - | - | - | - | - | - |
| VCR              | EUCAST   | 0.125 | 0.125 | IE | IE | IE | IE | 0.125 | 0.125 | 0.125 | 0.125 |
|                  | CLSI     | 0.125 | 0.5 | 0.5 | 1 | 0.125 | 0.5 | 0.125 | 0.5 |

SDD = susceptible dose dependant; IE = insufficient evidence.

### Table 3

**EUCAST antifungal breakpoints for *Aspergillus***

| Antifungal agent | *A. flavus* | *A. fumigatus* | *A. nidulans* | *A. niger* | *A. terreus* |
|------------------|-------------|----------------|--------------|------------|--------------|
|                  | S ≤ | R > | S ≤ | R > | S ≤ | R > | S ≤ | R > | S ≤ | R > |
| Amphotericin B   | - | - | 1 | 2 | - | - | 1 | 2 | - | - |
| Itraconazole     | 1 | 2 | 1 | 2 | 1 | 2 | - | - | 1 | 2 |
| Posaconazole     | - | - | 0.125 | 0.256 | - | - | - | - | 0.125 | 0.256 |
| Voriconazole     | - | - | 1 | 2 | - | - | - | - | - | - |
agreements (CA). The latter depends on the existence of interpretative break points. Of note, the laboratory should perform tests strictly as instructed in the commercial guidelines to get reliable results. Additionally, quality control strains, such as C. krusei ATCC 6258 and C. parapsilosis ATCC 22019 must be included in each commercial system batch, and be ascertain that all MIC values are within the expected ranges.

**Commercial broth microdilution methods**

SensiTite YeastOne® is a well-described colorimetric microdilution panel that contains dried serial twofold dilutions of up to ten antimycotics in individual wells. YeastOne® provides customizable dual-isolate five antifungal format for *Candida* spp. (clinical use), and single-isolate, nine antifungal, research-use-only format including anidulafungin and micafungin to be tested for yeast and filamentous fungi (not for use in diagnostic procedures). The susceptibility of isolates to antymycotics is assessed on the basis of growth or inhibition of the isolate in the culture media containing antymycotic agents. The system incorporates Alamar Blue®, a colorimetric indicator of an oxidation-reduction reaction (fungal growth changes media color from blue to pink). Endpoint determination was based on visual reading or software-facilitated visual reading (Vizion® system) after 24-25 h (*Candida* spp.) or 48-72 h (*Cryptococcus* spp.) of incubation at 35 °C. The panel has the advantage of being ready to use, easy to perform, quick and timely results, and individual packaging allows the test of one plate at a time. The Sensititre Yeast One® method showed good results in terms of reproducibility and agreement with reference methods considering fluconazole and *Candida* spp. (EA ≥ 95%) although a lower agreement (EA 79-92%) was found to *C. neoformans* isolates. YeastOne® panel was reported to yield higher MICs, in comparison with the CLSI method, for all drugs except for caspofungin and flucytosine. With respect to filamentous fungi, a strong correlation with the M38-A2 (CLSI) method was found for itraconazole and voriconazole. The method showed a strong correlation with CLSI to detect resistant isolates and may help to monitor the emergence of isolates with decreased susceptibility to antifungal agents.

Another commercially available system, called SensiQuattro *Candida* EU® (bestbiondx, Germany), correlates well with the antifungal clinical break points established by EUCAST. This 32-well colorimetric microdilution panel includes four doubling serial concentrations of amphotericin B, fluconazole, voriconazole, posaconazole, caspofungin, anidulafungin, micafungin, and flucytosine. The resulting colors are interpreted as follows: a yellow/orange color indicates yeast growth; a red color indicates yeast growth inhibition. When compared to the EUCAST reference, the broth microdilution method showed a good correlation for amphotericin B and azoles, but poor for echinocandins.

Vitek 2® yeast susceptibility test (bioMérieux, Inc.) is an automated method of yeast species identification and antifungal susceptibility testing through the analysis of yeast growth. The system provides 64-well cards containing aliquots of amphotericin B, fluconazole, flucytosine, and voriconazole in a miniaturized version of the broth dilution method. The system integrates a software program which validates and interprets susceptibility test results according to CLSI clinical breakpoints based on the drug MIC values. The high level of standardization achieved by this automated system was demonstrated in several studies. The number of hours to deliver an MIC result was reported to vary from 9.1 h to 15 h for *Candida* species (minimum 7.5 h to maximum 18 h) and 12.1 h for *C. neoformans*. In general, the MICs obtained by the Vitek 2® system are slightly higher than those generated by the CLSI methodology for both *Candida* and *Cryptococcus*. However, Vitek 2® results are reproducible, accurate, present a strong correlation with those obtained with the CLSI and the AFST-EUCAST reference methods for fluconazole, amphotericin B, flucytosine, and voriconazole. The correlation with the reference methods was also very good when resistance to antifungals was studied.

**Commercial agar-based methods**

Commercially prepared strips are available from bioMérieux (Etest®) and Liofilchem Diagnostici (MIC Test Strip®). The method consists of a predefined gradient of antifungal drug concentrations on a plastic strip that is used to determine the MIC. When the strip is applied on an inoculated agar surface, the antifungal agent is immediately transferred to the agar matrix and after an incubation time, an inhibition ellipse centered along the strip is formed. The recommended agar is RPMI 1640 supplemented with 2% glucose, prepared with MOPS in a 1.5% agar base. The Etest® provides strips with fluconazole, itraconazole, amphotericin B, flucytosine, voriconazole, posaconazole, and caspofungin. The MIC Test Strips® contains the same antifungal drugs plus anidulafungin, micafungin and ketoconazole. The incubation time range from 24-48 h for *Candida* species, from 48-72 h for *C. neoformans*, and for filamentous fungi it lasts 16 h or longer depending on the fungus’ genus. The MIC is read directly from the scale at the point where the edge of the ellipse intersects the strip. However, it is important to consider that, as for any test evaluating the antimicrobial susceptibility, the medium formulation and, in this case, the depth of the agar can strongly influence MIC results. Therefore, the manufacturer’s recommendations should be strictly followed to obtain MICs using strips.

Results obtained by the E-test method shows a > 71% correlation with those obtained by the AFST-EUCAST method. In both methods, the CLSI and EUCAST AFST, the agar-based E-test has been proposed as a more sensitive technology to discriminate strains of *Candida* species with *fks* mutations from wild-type (WT) strains by virtue of much higher MIC results observed in mutant strain. Considering *Cryptococcus*, the overall agreement level using the E-test MICs and the EUCAST AFST-MICs seems to be higher for voriconazole, fluconazole, itraconazole and flucytosine, than for amphotericin B, which has the lowest level of agreement. Regarding filamentous fungi, the agreement is higher for itraconazole than for amphotericin B, and the E-test method showed a good correlation with the CLSI M38-AFST one to detect *Aspergillus* resistance. Systematic comparisons between MIC results from reference laboratories and routine results obtained using commercially available methods could be more representative than the current practice to perform quality control with a specific set of reagents using a limited number of isolates.

**CONCLUSIONS**

The role of microdilution methods seems to be restricted to reference laboratories because they are laborious. In addition, the microbroth format is not commonly used in clinical laboratories. Several automated or semi-automated commercial methods based on agar diffusion or the use of colorimetric indicators in Etest, Sensititre YeastOne, Fungitest or Vitek have been designed for routine daily practice. Disk and strip diffusion
methodologies are simple, rapid, cost-effective and produce similar results to the reference methods for yeasts. Automated systems significantly reduce the biologist hands-on time, turnaround time, and variability due to the standardized format. Evaluation of these methodologies requires the determination of break point category agreements with reference methods. It is noteworthy that interpretative break points are only available for a few species of Candida, Cryptococcus and Aspergillus. Most of these tests have been able to detect in vitro resistance of Candida isolates, however some discrepancies have also been described. Reference procedures are irreplaceable nowadays to test and validate new antifungal agents, new methods and techniques, and the susceptibility profile of rare species which have not been evaluated by other methods\textsuperscript{27,26,41}. Also, the increase of resistant strains associated with treatment failure highlights the need of antifungal resistance surveillance, which should ideally be made in reference laboratories using reference procedures.

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