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CLINICAL MICROBIOLOGY - REVIEW

Factors influencing susceptibility testing of antifungal drugs: a critical review of document M27-A4 from the Clinical and Laboratory Standards Institute (CLSI)

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Abstract

Due to the increasing numbers of fungal infections and the emergence of drug-resistant fungi, optimization and standardization of diagnostic methods for the measurement of antifungal susceptibility are ongoing. The M27-A4 document by the US Clinical and Laboratory Standards Institute (CLSI) is presently used for the interpretation of minimum inhibitory concentrations of major opportunistic yeast species as measured by broth microdilution testing in many countries. Although microdilution is considered a benchmark for reproducible and accurate results, increased testing capacity, and limited human bias, the method is often inaccessible to routine clinical laboratories and researchers, especially in low-income countries. Furthermore, several studies suggest that there are still a considerable number of factors that make the estimation of in vitro activity of antifungal agents challenging. This review article summarizes the limitations of the M27-A4 standard which, despite the advances and improvements obtained by the standardization of antimicrobial resistance testing methods by CLSI, still persist.

Keywords CLSI · Yeast · Microdilution · Susceptibility · Antifungal drug

Introduction

Invasive fungal infections are often associated with significant morbidity and mortality, a problem which is aggravated by the need for sophisticated diagnostic approaches and therapeutic schemes [1, 2]. Despite improvements of diagnostic tools and therapeutic options [3–6], fungal infections are a major cause of high morbidity and mortality rates in predominantly immunocompromised human patients, with *Candida*, *Cryptococcus*, and *Aspergillus* genera being the main etiological agents of these infections [7, 8]. In immunocompromised patients, mycoses generally behave as opportunistic infections, causing invasive and often fatal disease [9].

Indeed, the availability of new antifungal drugs has given clinicians more options in recent years. There has been an increased use of these substances, not only for the treatment of diagnosed fungal infections but also for prophylactic, empirical, or preventive treatment [7]. However, due to the frequent and prophylactic use of these antifungal agents, numerous fungal strains have become resistant to the only drugs currently available, which are limited to only three major classes of antifungal drugs, i.e., azoles, polyenes, and echinocandins [10]. The increasing resistance as recorded for some species against antifungal agents is presently considered a serious health problem worldwide and the treatment of fungal infections is challenging due to the limited number of drugs available for clinical use as well as due to drug interactions and associated toxicities of certain classes of antifungals that may limit the therapeutic success [11].

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Because yeast infections are often associated with high mortality and there are repeated reports on therapeutic failures associated with the isolation of resistant strains, there is an ongoing need for the measurement of antifungal susceptibility as an important determinant of therapeutic success [12]. Currently, the M27-A4 standard of the North American Clinical and Laboratory Standards Institute (CLSI) guides measurement and interpretation of the minimum inhibitory concentrations (MIC) of important antifungal drugs which are used against different yeast species like *Candida* spp. and *Cryptococcus* spp. The protocol recommends the plaque microdilution method. Thereby, colony suspensions in pH 7.0 buffered RPMI-1640 broth with MOPS (3- (N-morpholino) propanesulfonic acid) are analyzed with 10 concentrations of each antifungal drug. After incubation at 35 °C for 72 h for *Cryptococcus* spp., or for 24 h for *Candida* species, visual assessment of MIC is performed in comparison with a control well containing antifungal-free inoculum to support fungal growth [13].

Although this approach is considered a reference standard for antifungal susceptibility testing, there are proposals suggesting modifications of the assay, especially regarding the chosen (i) growth medium, (ii) culture medium buffer, (iii) inoculum concentration, (iv) incubation time, and (v) way of assessing the result. In addition, there are suggestions for protocol changes in order to improve the method [14–17] and proposals for a microdilution assay that can be applied in resource-limited environments [18]. In this review, limitations of the M27-A4 standard as perceived by the authors are presented and discussed.

Determination of antifungal susceptibility

The development of standardized reference methods for antifungal susceptibility testing occurred in response to the increasing number of available antifungal agents and to the high incidence of systemic fungal infections [19]. Such reference methods combined with the outcomes of clinical assessments allow the identification of minimum inhibitory concentrations (MIC) at which the fungus can be considered susceptible, susceptible in the case of a higher dose or resistance [20].

Generally available techniques for the in vitro evaluation of antifungal activity comprise agar dilution, agar diffusion, and broth dilution methods. The principle of these methods is the exposure of a definite inoculum of the microorganism to defined concentrations of the analyzed drug under optimal growth conditions. Thereby, it is observed whether fungal growth is minimized or not. The final readout of the dilution tests in liquid media or on solid media will allow the identification of the lowest concentration of the drug that still inhibits the growth of a given microorganism [21].

The Clinical and Laboratory Standards Institute (CLSI) and the European Committee on Antimicrobial Susceptibility Testing (EUCAST) have published the two most widely used guidelines worldwide for the testing of susceptibility of yeasts by broth microdilution, i.e., the documents "M27-A4" and "E. Def. 7.3.2", respectively [13, 22]. Both are similar but show methodological and interpretative differences in breakpoints [23], adapted to the dosages of the antimicrobial drugs as distributed in the regions where they are applied. In this context, a major challenge is the definition of harmonized cutoff points, as this would also require international standardization of drug dosage and licensing. Europe and several other regions adhere to the EUCAST recommendations. The EUCAST guidelines are generally freely available on the committee's website, a fact that might make them more attractive compared with the protocols marketed by the CLSI [24].

Kassim et al. [23] report a number of disadvantages of CLSI protocols for antimicrobial susceptibility testing in comparison with the EUCAST, in detail: (i) the cost of obtaining the CLSI guidelines of up to $500 for non-members of the Institute, or $350 for members of a three-document package; (ii) the influence that the Food and Drug Administration (FDA) has on determining the MIC and this also raises major concerns about the influence of the pharmaceutical industries in setting the guidelines; (iii) the CLSI voting committee, which is constituted by representatives from both academia and the profit sector; (iv) ignorance regarding antibiotics and/or antifungals which are not registered in the United States in the CLSI guidelines; and (v) inaccessibility of many details about the decision-making process to the public.

Regardless of their undenied advantages, broth microdilution as the reference method for antifungal sensitivity testing is time-consuming and laborious for routine clinical and research laboratories. For this reason, commercial methods are frequently used that correlate more or less well with reference methods such as E-test® (bioMérieux, Marcy-l'Étoile-França), Sensititre® (Thermo Fisher Scientific, Waltham-EUA), and Vitek2® (bioMérieux, Marcy-l'Étoile-França), including manual, semi-automated, and automated approaches which do not require complex manual handling. In most instances, clinical decisions based on these alternative methods show good clinical correlation compared with antifungal in vitro susceptibility testing based on the reference standard [7]. Regardless of the method, the results should be carefully interpreted, because the identification of the minimum inhibitory concentration (MIC) can be challenging. There are also host factors involved in the clinical response to an antifungal therapy and there is not always a satisfying correlation between the MIC and clinical outcome [25].

Broth microdilution: M27 standard

The broth macrodilution method was the first thoroughly optimized and standardized method for the testing of antifungal

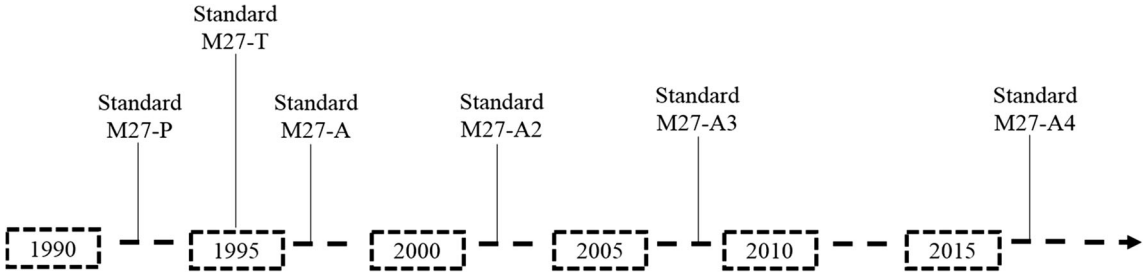

Fig. 1 Standards developed by the CLSI for broth dilution testing for the determination of susceptibility of yeasts to various antifungal drugs

susceptibility in yeasts. It was developed by the CLSI, formerly NCCLS (National Committee for Clinical Laboratory Standards), which is an entity responsible for the standardization of clinical laboratory techniques in the United States (US). The first version appeared in 1992 as document M27-P (Fig. 1). It was later adapted to allow microdilution in plates in order to test higher numbers of isolates. Prior to the development of this method, antifungal susceptibility studies showed inconsistent results and were non-reproducible, because there were many factors that influenced the applied tests. Therefore, in response to the requests by the medical and scientific community, the NCCLS established a subcommittee in 1985 that was responsible for analyzing the different variables involved in in vitro susceptibility testing of yeasts incubated with antifungal agents. In the same year, after publishing its first report, the CLSI concluded that it would be useful to develop a more reproducible methodology for benchmarking purposes [19].

Within the CLSI report, there was an agreement on various elements of the methodology of broth dilution. Firstly, due to examples of antagonism of substances within some complex culture media with certain antifungal agents, the chosen media should be defined in detail. Secondly, standardization of work steps was focused on yeasts as these fungi were the predominant cause of fungal infections. However, additional studies were needed to address the issues like details of the inoculum preparation and volume, the culture medium, the incubation time, the incubation temperature, and the determination of the MIC breakpoint.

As part of the strategy to find a method with a high reproducibility of results, several multicenter studies were performed in order to define the optimal assay conditions and were summarized in the M27 standards. Table 1 presents the most important studies which allowed the preparation of the CLSI M27-P standard by providing data which allowed the standardization of antifungal susceptibility testing of yeasts.

As a result of the mentioned collaborative studies by the subcommittee, the CLSI published a reference method for determining the sensitivity of *Candida* spp. in liquid medium in 1992, which resulted in the proposed standard M27 (M27-P) and which was also called broth dilution or macrodilution technique. Over the next 4 years, i.e., from 1992 to 1996, the subcommittee focused on establishing MIC reference value ranges for two quality control strains. After several studies evaluating the equivalence of results obtained by macrodilution versus microdilution, it was possible to introduce a microdilution testing scheme which provided results corresponding to the macrodilution reference test. This microdilution procedure was included in the revised standard published in 1995 (M27-T).

In a later review of the document, the CLSI developed "cutoffs" relevant to the antifungal agents available at this time (amphotericin B, 5-fluorocytosine, and azole derivatives) and included these cutoffs in the version M27-A

Table 1 Studies that contributed to the preparation of the standard M27-P

Researcher/ year	Factor investigated	Result	Reference
Pfaller et al., 1988	Preparation of the yeast inoculum	Defined spectrophotometric method	[26]
Pfaller et al., 1990	Selection of the culture medium (YNB, SAAMF, RPMI-1640, or high-resolution broth) and the incubation temperature optimum	Defined medium: RPMI-1640Defined temperature: 35 °C	[27]
Fromtling et al., 1993	Incubation time, inoculum concentration, and criteria for the definition of MIC	Incubation time: 48 hInoculum concentration: 2.5×10^3 cells/mLDefined interpretation criteria	[28]
Espinel-ingroff et al., 1992 Barchiesi et al., 1994	Comparison of macrodilution and microdilution	Acceptance of the microdilution methodology (equivalence of the results between the methods)	[29, 30]
Pfaller et al., 1995	Quality control with ATCC strains	Establishing of *Candida parapsilosis* and *Candida krusei* as ATCC control strains	[31]

in 1997. Since then the subcommittee focused on the development of 24- and 48-h MIC reference ranges for amphotericin B, 5-fluorocytosine, fluconazole, ketoconazole, itraconazole, posaconazole, ravuconazole, and voriconazole with the aim of facilitating interlaboratory agreement of sensitivity testing to antifungal agents for yeasts [32]. In 2002, the results of this study were included in the second edition of the standard called M27-A2. The standard was later revised again, resulting in the up-to-date version M27-A3 [19], which was published in 2008 as the 3rd edition of the document. This version included MIC values for antifungals from the echinocandin class, i.e., caspofungin, micafungin, and anidulafungin, and with the exception of this new class, an assessment of the final reading point after 48 h of incubation was preferred for *Candida* spp. Consequently, the M27-A3 standard provided MIC ranges for readings after 24 and 48 h.

In 2017, this document evolved to version M27-A4 [13], and an evaluation of the final reading point for the in vitro susceptibility testing of *Candida* spp. with selected antifungal agents was defined for the 24-h incubation time. The new informational supplement to the standard, the CLSI M60 document [33], details the most up-to-date standards for antifungal susceptibility testing of yeasts and contains interpretative categories and MIC ranges (24 h) for the organization of quality control procedures. In addition, in order to facilitate the reading of the standard, this version brought a change in the format and arrangement of the document. In short, the processes are now described as flows and tables.

Despite the advances and improvements achieved for the standardization of antifungal resistance testing methods as suggested by the CLSI, some limitations still persist, such as (i) the unreliable detection of *Candida* spp. resistance against amphotericin B, (ii) the growth and simplification of the interpretation of growth for *Cryptococcus* spp. that needs to be improved, and (iii) the reaction endpoint for fluconazole that needs to be more clearly defined [19]. While many publications demonstrate the need for revision in order to further improve microdilution testing, the latest protocol update focusing on factors such as culture media, buffer, pH, inoculum, and interpretation schemes occurred nearly 30 years ago in 1992, when the subcommittee presented the proof-of-principle studies and these provided data for the standardization of yeast susceptibility testing to antifungal agents.

Clinical mycology in many countries of the world is based on this susceptibility testing method in order to define the agent of choice for an individual fungal infection and in order to monitor the local and global epidemiology of antifungal resistance [7]. The broth microdilution method, as reported in the CLSI document M27-A4, is now a widely accepted reference. However, the selection of the appropriate drug expands on the one hand and improvements of the test protocol are desirable on the other hand [34].

Proposed modifications to CLSI M27-A4

In the 1990s, many modifications of the M27-P standard were implemented in response to specific problems. Some of these modifications have been described in the standard M27-A3 [19] in a "special circumstances modification" table with corresponding bibliographic references. This includes the use of antibiotic medium 3 to support the evaluation of the effects of amphotericin B on the growth of *Candida* spp. [35], the use of yeast nitrogen base (YNB) to better visualize the growth of *C. neoformans* and to improve the clinical interpretability of MICs against various antifungals [36, 37], and the supplementation with glucose to achieve a final concentration of 20 g/L in order to improve the growth of the inoculum [38]. The usefulness of each modification was not confirmed by the committee and the modifications were provided only as a reference for laboratories interested in studying adaptations of the M27-P standard, not as part of the methodology. Now, in the updated M27-A4 version of the standard, this information has been removed [13].

The inclusion of variations of the present CLSI protocol has also been suggested by other authors. For example, Vitale and colleagues [14] evaluated the influence of the size of the *C. neoformans* polysaccharide capsule on the in vitro activity of five antifungal agents (amphotericin B, fluconazole, voriconazole, itraconazole, 5-fluorocytosine). The capsule formation was induced by adding $NaHCO_3$ to the medium and then incubating the cells in an atmosphere containing 5% CO_2. Fluconazole was associated with higher MICs after capsule induction. Accordingly, the authors concluded that the determination of antifungal activity after capsule induction is of clinical relevance, as the correlation between in vitro results and clinical outcome may be affected. The authors suggested adding this induction step of capsule formation in the CLSI M27-A3 standard for antifungal microdilution testing as it influences the in vitro activity of antifungal drugs.

Similarly, Cordoba et al. [15] demonstrated that the size of the polysaccharide capsule produced by *C. neoformans*, which is if *C. neoformans* grows in a cellular environment in vivo than if it grows under normal laboratory conditions, influences the in vitro susceptibility against antifungal drugs. In general, isolates with larger capsules were more resistant to amphotericin B compared with isolates with smaller capsules in this study.

Garcia-Effron and colleagues [16] demonstrated that the addition of bovine serum albumin (BSA) to the protocol suggested by the CLSI document M27-A3 improves the detection of echinocandin resistance in *Candida* spp. In vivo, this class of drug is strongly bound to serum proteins. The test was performed in line with the CLSI M27-A3 method with 50% (w/v) serum and different BSA concentrations (ranging from 2.5 to 100 mg/mL). The authors concluded that RPMI-1640 medium with 50 mg/mL bovine serum albumin was more

reliable than RPMI-1640 alone in detecting resistant *Candida* spp. isolates. Accordingly, the addition of this protein to RPMI-1640 could be considered a useful modification of the echinocandin susceptibility testing scheme by the CLSI. However, optimal test conditions have to be established yet and the proof of reproducibility in studies with various laboratories is essential in order to propose such a modification.

The impact of the pH value on the antifungal susceptibility of *Candida albicans* isolated from patients with vulvovaginal candidiasis was investigated by Liu and colleagues [39] using the broth microdilution method (CLSI, document M27-A2). Antifungal susceptibility testing was performed at pH 7.0 and at pH 4.0. The minimum inhibitory concentrations of antifungals for *C. albicans* at pH 4.0 were significantly higher than those at pH 7.0 (0.25 vs 0.03 μg/mL for miconazole, 0.50 vs 0.03 μg/mL for clotrimazole, 0.50 vs 0.25 μg/mL for fluconazole, and 32 vs 2 μg/mL for nystatin, respectively). The authors recommended that growth media at different pH values should be used for susceptibility testing depending on the isolation site of *C. albicans*.

Zaragoza et al. [40] demonstrated that the growth of *C. neoformans* and other non-fermenting yeasts can be improved by adding new factors to the protocols suggested by the CLSI and EUCAST. The authors concluded that MIC assessment without an overestimation of MIC values can be facilitated by altered test conditions comprising the use of the medium YNB, plate shaking, readout after the 48-h incubation, an inoculum size of 10^5 cells/mL, and incubation at 30 °C.

Further, Rodriguez-Tudela et al. [17] verified that agitation favored the growth of *Cryptococcus neoformans* regardless of the medium used (RPMI-1640, RPMI-1640 with 2% glucose, and yeast nitrogen base (YNB)), while growth in static cultures was very poor in this study. The authors recommended that incubation under agitation should be included in future optimization and updates of antifungal susceptibility testing. In particular, they suggested this approach for *C. neoformans*. Until today, agitation is described as merely optional in the standard protocol.

Factors influencing the minimum inhibitory concentrations (MICs)

Growth medium

Fungi are eukaryotic cells. Accordingly, they belong to the most complex and evolved microorganisms. Not surprisingly, no individual culture medium is sufficient to isolate and cultivate the whole spectrum of clinically relevant fungi [41, 42]. The availability of essential nutrients in a culture medium such as glucose and nitrogen compounds determines the growth rates of yeast cells. Fungi are able to adjust their growth and development to available nutrients; they can also adapt to nutritional depletion by activation of alternative metabolic mechanisms [43].

Similar to other living organisms, yeasts require minerals; in particular, they depend on a supply of potassium, iron, magnesium, manganese, calcium, copper, and zinc. Many other nutrients are known to stimulate yeast growth. They comprise pantothenic acid, inositol, nicotinic acid, thiamine, p-aminobenzoic acid, and pyridoxine. However, these substances are not essential for the growth of yeast cells. *Candida albicans*, for example, is able to replicate in ammonium salt media as the only source of nitrogen, although amino acids are the preferred nitrogen source in case of availability. Different metabolic pathways can be activated sequentially, thus reflecting the potential and the specificities of the permeases located in the cellular membrane of yeasts [44].

These fungal microorganisms are capable of synthesizing all 20 proteinogenic amino acids, including nine that are essential for humans, and must be extracted from the diet in the human gut, i.e., phenylalanine, valine, threonine, tryptophan, isoleucine, methionine, leucine, lysine, and histidine. This is because the enzymes that catalyze particular stages of the biosynthesis of amino acids essential to humans are fungus-specific, as fungi have developed their own biosynthesis pathways [45]. The study by Lee and colleagues [46] demonstrated that a synthetic amino acid medium is developed based on the aminopeptidase profile by *C. albicans* and is composed of only six amino acids, biotin, inorganic salts, and glucose, supporting optimal growth of this microorganism.

Nowadays, antifungal susceptibility testing is performed with RPMI-1640 medium, a glutamine-free, bicarbonate-free medium containing a phenol red indicator and buffered with 0.165 3-(N-morpholino-) propanesulfonic acid (MOPS) mol/L. It is named by the Roswell Park Memorial Institute. This medium is a modification of medium 5A introduced by McCoy and colleagues who described the so-called Novikoff hepatoma as a liver tumor requiring 12 amino acids and glutamine for in vitro growth [47]. It was later modified to be used for the growth of human cells as a chemically defined medium. It is useful of supporting rapid cell growth and is also considered one of the best multiple-purpose media for culturing various types of mammalian cells in vitro [48].

As suggested by the M27-P standard, the RPMI-1640 medium has been considered appropriate for antifungal susceptibility testing. It has been chosen from the study by Pfaller et al. [27], because it was associated with the highest level of interlaboratory agreement. However, the authors stated that its suitability was not significantly different from the suitability of yeast nitrogen base (YNB) medium. Further, the use of YNB at pH 7 and supplemented with glucose at a final concentration of 0.5 g/L is an alternative option. It is described in the CLSI M27-A3 standard to facilitate *C. neoformans* growth and to improve the clinical impact of MICs of antifungal agents [36].

According to Radetsky et al. [49], the advantages of RPMI-1640 medium are that it is readily available, inexpensive, available as a quality-controlled product, chemically defined, simple to prepare, and buffered by carbonate and phosphate and that it contains the pH indicator phenol red. However, with regard to inexpensive and easy preparation, it is clear that this reference [49] is outdated. Moreover, even when using RPMI-1640, one of the major challenges of the presently suggested M27-A4 methodology is the reliable identification of amphotericin B resistance in *Candida* spp. isolates. For this purpose, the studies cited below tried to identify reliable alternatives to the present standard.

In the 1990s, Rex and colleagues [50] performed experiments to identify alternative media and pH conditions allowing the reliably identification of amphotericin B-resistant isolates. They concluded that the buffered antibiotic medium 3 at pH 5 or pH 7 led to the most reliable results among the assessed media Sabouraud broth, antibiotic medium 3, Casitone broth, and YNB. The use of antibiotic medium 3 allowed the identification of a series of resistant isolates already within the first 24 h of incubation. Lozano-Chiu et al. [35] also concluded that the use of antibiotic medium 3 broth could facilitate in the detection of antifungal resistance. However, as antibiotic medium 3 shows substantial variability between different batches, this medium has not been standardized for antifungal susceptibility testing so far.

In order to identify alternatives to the use of the difficult-to-standardize antibiotic medium 3, Cuenca-Estrella and colleagues [51] analyzed and compared the results of the MICs obtained using the semi-defined Iso-Sensitest broth with the MICs obtained with antibiotic medium 3 and RPMI-1640 broth. The authors described that MICs recorded by incubation in Iso-Sensitest broth were highly reproducible and reliably identified *Candida* spp. with resistance against amphotericin B. Cuenca-Estrella and colleagues concluded that the most pronounced discrimination between susceptible and resistant isolates was achieved by using Iso-Sensitest supplemented with glucose as the test medium and by subsequent spectrophotometric readout after 24 h of incubation.

Despite the result of these studies, nothing has changed and the M27-A4 standard still recommends RPMI-1640 as the growth medium of choice. In addition, the lack of agreement on supplementation of RPMI-1640 medium to achieve a final glucose concentration of 20 g/L as well as on the use of YNB to facilitate *C. neoformans* growth and to improve the clinical meaningfulness of antifungal MICs remains an important limitation of the present M27-A4 standard.

In addition to those mentioned above, there are few studies in the up-to-date literature on the suitability of other growth media for antifungal susceptibility testing. The present scientific work is more focused on the establishing of cutoffs for available antifungal drugs and also on the standardization of reference assays for fungi that are not included in the current CLSI standard such as *Paracoccidioides* spp., *Histoplasma* spp., and *Malassezia pachydermatis* [52–54]. Due to this prioritization, other important fields of diagnostic research like defining an ideal growth medium that is easy to prepare and inexpensive and that more reliably detects resistant isolates in order to improve the clinical meaningfulness of MICs of the assessed antifungal drugs remain out of scope.

Buffer solution for the culture medium

The use of buffer solutions in order to maintain the pH value within a targeted range is a very common practice in chemical, biochemical, and biological approaches [55]. MOPS 3-(N-morpholino) propanesulfonic acid is a buffer developed for broad use in biochemistry. With a pKa of 7.15 at 20 °C, it is very useful for buffering biological systems and assays requiring a neutral pH value [56].

According to the CLSI [19], the buffer solution MOPS is considered useful for antifungal susceptibility testing, as it is compatible with antifungals without showing antagonizing or interacting effects on them. However, there is no description of any reference for this choice in the present standard. The study by Pfaller et al. [27], which defined the ideal medium for the presently applied methodology, did not compare buffer solutions other than MOPS in the growth media. All tested media were buffered with MOPS only at pH 7.0. Accordingly, it is not possible to decide which buffer might really be the best one for antifungal susceptibility testing.

Two relatively old studies compared sodium bicarbonate, MOPS-Tris, and phosphate buffer as alternative buffer systems at varying costs for antifungal susceptibility testing. One study was from Gadea and colleagues [57], comparing the effect of buffer systems on in vitro activity of amphotericin B, 5-fluorocytosine, fluconazole, itraconazole, and ketoconazole as assessed by microdilution including 93 yeast isolates. The authors concluded that susceptibility testing was of comparable reproducibility and the results could be easily interpreted both manually and spectrophotometrically regardless of the buffer system used with the exception of *C. parapsilosis* and *Cryptococcus neoformans*. These species did not grow properly under any of the conditions tested, which were (i) RPMI-1640 with 2% glucose buffered with 0.165 M MOPS at pH 7.0, (ii) RPMI-1640 under the same conditions but buffered to pH 7.4, and (iii) RPMI-1640 with 2% glucose buffered to pH 7.4 with 0.15% sodium bicarbonate.

The second study was from McIntyre and Galgiani [58], who used SAAMF (synthetic amino acid medium fungal) culture medium for the assessment of the antifungal activity of cilofungin and observed that there were no variations in the measured susceptibility results for the different buffer systems used: HEPES, MOPS, and a combination of MOPS-Tris and phosphate.

Inoculum concentration

Pfaller et al. [26] performed a comparative study applying four inoculum preparation methods (spectrophotometric, Wickerham card, hemocytometer, and Prompt inoculation system). They concluded that the spectrophotometric method should be chosen for the preparation of a yeast inoculum, because this approach showed higher reproducibility and less variability. Fromtling and colleagues [28] defined that the optimal inoculum size for susceptibility testing was 2.5×10^3 cells/mL.

In line with this, the preparation of the inoculum for susceptibility testing according to protocol M27-A4 is as follows: (1) sub-culturing of yeasts should be performed in sterile tubes with Sabouraud dextrose agar or potato dextrose agar to ensure purity and viability of the strain. The incubation temperature should be 35 °C. (2) The inoculum should be prepared using five colonies with a diameter of ~ 1 mm after incubation for 24 h for *Candida* species or after incubation for 48 h for *C. neoformans*. The colony material should be suspended in 5 mL of 0.145 mol/L sterile saline (0.85% saline). (3) The resulting suspension should be vortexed for 15 s and the cell density should be adjusted with a spectrophotometer, adding sufficient saline to obtain transmittance equivalent to that of a McFarland 0.5 standard solution with a wavelength of 530 nm. The standard yeast suspension should be vortexed for 15 s, diluted 1:50 and then 1:20 with the culture medium to get the 2 × concentrated inoculum applied in the test (1×10^3 to 5×10^3 cells/ mL). The inoculum (2 ×) will then be diluted 1:1 by inoculation into the microtiter plate to reach the final concentration (0.5×10^3 to 2.5×10^3 cells/mL) [19].

As early as in the 1990s when the CLSI subcommittee investigated the variables affecting fungal susceptibility testing of antimycotics, Ghannoum et al. [36] studied a number of ideal conditions for determining *Cryptococcus neoformans* susceptibility to antifungal agents and proposed microdilution using 10^4 cells per mL as final inoculum with YNB (pH = 7) as growth medium and 48 h as incubation period as a simple, accurate, and reproducible method for susceptibility testing of *C. neoformans* using fluconazole, amphotericin B, and 5-fluorocytosine.

Rodriguez-Tudella and colleagues [17] examined three media recommended by the CLSI protocol M27-A3 (RPMI-1640; RPMI-1640, 2% glucose; and YNB) and described that inoculum of 10^5 cells/mL produced optimal growth for *C. neoformans*, thus demonstrating a need for revision of the Northern American reference test.

Further, Cuenca-Estrella and co-workers [51] reported that susceptibility testing performed with antibiotic medium 3 using 10^3 cells/mL inoculum was able to distinguish between resistant and susceptible *Candida* spp. isolates. These authors also verified that factors like the readout method, the incubation time, or glucose addition showed no significant effect on the recorded minimum inhibitory concentrations. However, a larger inoculum (10^5 cells/mL) interfered with the measured MIC values.

Incubation time

Fromtling et al. [28] were able to show that fungal growth in microdilution assays at 24-h incubation time was uniformly poor in all assessed media and there was no reproducibility. In contrast, incubation for 48 h showed comparable growth with low standard deviation values. As a conclusion, the results from MIC assessment at 48 h (72 h for *C. neoformans*) are in better agreement with the reference method broth macrodilution than after 24-h incubation time.

In line with this, the M27-A3 methodology for *Candida* spp. recommends endpoint assessment after 48 h with the exception of echinocandins. The M27-A3 standard does not make it clear, however, whether the results obtained after 48 h of incubation are more appropriate than those obtained after 24 h, or vice versa, for clinical interpretation purposes [19]. The growth of the inoculum defines the final incubation time for MIC assessment.

Thus, Arthington-Skaggs and colleagues [59] suggested the inclusion of assessments after 24 h of growth, because (i) it is often possible to evaluate MIC results at this incubation time and (ii) such early readout may be clinically relevant in the case of some isolates and patients. According to the authors, isolates for which early assessment is important are those with a dramatic increase in MIC from 24 to 48 h of growth. This can occur due to significant residual growth, which is defined by only partial inhibition of growth over a wide range of increasing concentrations of fungistatic drugs, a phenomenon which is also known as "trailing." Trailing is estimated to be observed for approximately 5% of fungal isolates, so isolates that seem susceptible after 24 h of growth show growth patterns suggesting resistance after 48 h of incubation.

Ostrosky-Zeichner et al. [60] also justified reading MICs of fluconazole after 24 h instead of 48 h when testing *Candida* spp. by the standard CLSI M27-A2 method. Except for isolates in the dose-dependent susceptible category, such as *Candida glabrata*, the data analysis showed good correlation between MICs obtained after 48 h and after 24 h next to comparable results and parameters of efficacy. According to the authors, it was necessary to define the interpretive interruption point at 24 h, as it would provide clinically useful information for doctors who treat patients with invasive candidiasis.

Accordingly, the most recent version of the standard, CLSI M27-A4, states that for broth microdilution, the QC limits are now set for all antifungal agents (including echinocandins) at 24 h (M60) [33]. Accordingly, for *Candida* spp., broth microdilution MICs should be read at 24 h of incubation (at

72 h for *Cryptococcus* spp.). However, it remains unresolved whether or not growth media, for which there is not enough growth in the control well, can be kept for another 24 h [13].

Readout results

The M27-A4 document recommends that microdilution plates should be incubated at 35 °C for 24 h for *Candida* spp. (for 72 h for *Cryptococcus* spp.) and that the presence or absence of growth should be visually assessed. After that, the microdilution plate wells shall be scored and the growth in each well shall be compared with the growth control well. Thereby, a mirror can facultatively be used. Each well of the microdilution plate is evaluated applying a numerical scale: 0 = optically clear, corresponding to 100% growth inhibition; 1 = slightly hazy, corresponding to 75% growth inhibition; 2 = prominent decrease in turbidity, corresponding to 50% growth inhibition; 3 = slight reduction in turbidity, corresponding to 25% growth inhibition; 4 = no reduction in turbidity corresponding to 0% growth inhibition [13].

For amphotericin B, the reaction endpoints are clearly defined and it is easy to identify the MIC as the lowest antifungal concentration which prevents any discernible degree of growth. In general, poorly defined reaction endpoints are not seen for this drug. Accordingly, the amphotericin B MIC is defined as the lowest concentration at which the score 0 (optically clear) is observed. For the azoles, 5-fluorocytosine, and the echinocandins, the in vitro endpoints are less clearly defined than those described for amphotericin B, potentially resulting in substantial variability. The MIC of these substances is defined as the lowest concentration at which score 2 (prominent decrease in turbidity) or more reduction of growth is observed. This score corresponds to approximately 50% or more of growth inhibition, as numerically determined by a spectrophotometer [13]. Thereby, visual interpretation depends on the technician's experience [25], constituting a likely source of bias.

In line with this, the importance of automation using spectrophotometric devices was emphasized by the EUCAST, which proposes obligate spectrophotometric analysis for yeast testing. More than this, the EUCAST protocols include modifications of some CLSI parameters such as inoculum preparation and concentration as well as glucose supplementation in RPMI. Unlike CLSI, the EUCAST considers the breakpoint to be the well where there is absorbance (applying 530-nm filters) of 50% or less compared with the positive control. In addition, using kinetic procedures, antifungal MICs can be determined at any desired time during the incubation period, without defined 24- or 48-h periods required for the final assessment [61]. The EUCAST standard E. Def. 7.3.2 is currently considered as the European reference and widely used, even beyond the European borders [62].

Colorimetric assays are increasingly in use for antifungal resistance testing to ease manual analysis in routine laboratories, but the trailing problem is not controlled and the technicians must still be very well trained to perform the assessments reliably [63–65]. Tetrazolium salts and the "Alamar Blue®" dye (resazurin blue), an effective indicator of microbial growth, are frequently used in such tests [64, 66–68].

Conclusion

Although M27 has been the standard in use by many clinical laboratories to assist antifungal susceptibility testing since 1992, some aspects of the protocol are controversially discussed and leave room for improvement. In spite of many publications suggesting a need for reevaluation and improvements of the present standard, the introduction of modifications and the publishing of an updated protocol meet considerable difficulties. Accordingly, further multicenter studies should be implemented to ensure better evidence for the improvements that are proposed in the literature.

Compliance with ethical standards

Conflict of interest The authors declare that there are no conflicts of interest.

References

1. Albataineh MT, Sutton DA, Fothergill AW, Wiederhold NP (2016) Update from the laboratory: clinical identification and susceptibility testing of fungi and trends in antifungal resistance. Infect Dis Clin N Am 30:13–35. https://doi.org/10.1016/j.idc.2015.10.014
2. Spitzer M, Robbins N, Wright GD (2017) Combinatorial strategies for combating invasive fungal infections. Virulence 8:169–185. https://doi.org/10.1080/21505594.2016.1196300
3. Day JN, Chau TTH, Wolbers M, Mai PP, Dung NT, Mai NH, Phu NH, Nghia HD, Phong ND, Thai CQ, Thai LH, Chuong LV, Sinh DX, Duong VA, Hoang TN, Diep PT, Campbell JI, Sieu TPM, Baker SG, Chau NVV, Hien TT, Lalloo DG, Farrar JJ (2013) Combination antifungal therapy for cryptococcal meningitis. N Engl J Med 368:1291–1302. https://doi.org/10.1056/NEJMoa1110404
4. Nami S, Aghebati-Maleki A, Morovati H, Aghebati-Maleki L (2019) Current antifungal drugs and immunotherapeutic approaches as promising strategies to treatment of fungal diseases. Biomed Pharmacother 110:857–868. https://doi.org/10.1016/j.biopha.2018.12.009
5. Sanguinetti M, Posteraro B, Beigelman-Aubry C, Lamoth F, Dunet V, Slavin M, Richardson MD (2019) Diagnosis and treatment of invasive fungal infections: looking ahead. J Antimicrob Chemother 74:II27–II37. https://doi.org/10.1093/jac/dkz041
6. Wiederhold NP Antifungal resistance: current trends and future strategies to combat. Infect Drug Resist 10:249–259. https://doi.org/10.2147/IDR.S124918
7. Alastruey-Izquierdo A, Melhem MSC, Bonfietti LX, Rodriguez-Tudela JL (2015) Susceptibility test for fungi: clinical and

laboratorial correlations in medical mycology. Rev Inst Med Trop 57:57–64
8. Sun KS, Tsai CF, Chen SCC, Huang WC (2017) Clinical outcome and prognostic factors associated with invasive pulmonary aspergillosis: an 11-year follow-up report from Taiwan. PLoS One 12:1–10. https://doi.org/10.1371/journal.pone.0186422
9. Colombo AL, Tobón A, Restrepo A, Queiroz-Telles F, Nucci M (2011) Epidemiology of endemic systemic fungal infections in Latin America. Med Mycol 49:785–798. https://doi.org/10.3109/13693786.2011.577821
10. Perfect JR (2017) The antifungal pipeline: a reality check. Nat Rev Drug Discov 16:603–616
11. Martinez R (2006) Atualização no use of antifungal agents. J Bras Pneumol 32:449–460
12. Murray PR, Rosenthal KS, Pfaller MA (2014) Medical microbiology. Elsevier, Rio de Janeiro
13. Clinical and Laboratory Standards Institute (2017) Reference method for broth dilution antifungal susceptibility testing of yeasts. Approved standard-fourth edition. CLSI document M27-A4.Wayne, PA
14. Vitale RG, Pascuccelli V, Afeltra J (2012) Influence of capsule size on the in vitro activity of antifungal agents against clinical *Cryptococcus neoformans* var. *grubii* strains. J Med Microbiol 61:384–388. https://doi.org/10.1099/jmm.0.036152-0
15. Córdoba S, Afeltra J, Vitale RG (2011) Evaluation of the in vitro activity of amphotericin B by time-kill curve methodology against large and small capsulate *C. neoformans* isolates. Diagn Microbiol Infect Dis 71:260–262. https://doi.org/10.1016/j.diagmicrobio.2011.08.003
16. Garcia-Effron G, Park S, Perlin DS (2011) Improved detection of *Candida* sp. fks hot spot mutants by using the method of the CLSI M27-A3 document with the addition of bovine serum albumin. Antimicrob Agents Chemother 55:2245–2255
17. Rodríguez-Tudela JL, Martín-Díez F, Cuenca-Estrella M, Rodero L, Carpintero Y, Gorgojo B (2000) Influence of shaking on antifungal susceptibility testing of *Cryptococcus neoformans*: a comparison of the NCCLS standard M27A medium, buffered yeast nitrogen base, and RPMI-2% glucose. Antimicrob Agents Chemother 44:400–404
18. Smith KD, Achan B, Hullsiek KH, McDonald T, Okagaki LH, Alhadab AA, Akampurira A, Rhein JR, Meya DB, Boulware DR, Nielsen K, ASTRO-CM/COAT Team (2015) Increased antifungal drug resistance in Ugandan clinical isolates of *Cryptococcus neoformans*. Antimicrob Agents Chemother 59:7197–7204
19. Clinical and Laboratory Standards Institute (2008) Reference method for broth dilution antifungal susceptibility testing of yeasts. Approved standard-third edition. CLSI document M27-A3.Wayne, PA
20. Hazen KC (2013) Influence of DMSO on antifungal activity during susceptibility testing in vitro. Diagn Microbiol Infect Dis 75:60–63. https://doi.org/10.1016/j.diagmicrobio.2012.09.002
21. Sidrim JJC, Rocha MFG (2004) Medical mycology in light two contemporary authors. Guanabara Koogan, Rio de Janeiro
22. European Committee on Antimicrobial Susceptibility Testing (2020) Method for the determination of broth dilution minimum inhibitory concentrations of antifungal agents for yeasts. E. Def 7.3.2 (EUCAST-AFST)
23. Kassim A, Omuse G, Premji Z, Revathi G (2016) Comparison of clinical laboratory standards institute and European committee on antimicrobial susceptibility testing guidelines for the interpretation of antibiotic susceptibility at a university teaching hospital in Nairobi, Kenya: A cross-sectional stud. Ann Clin Microbiol Antimicrob 15:1–7
24. Kahlmeter G (2015) The 2014 Garrod lecture: EUCAST - are we heading towards international agreement. J Antimicrob Chemother 70:2427–2439. https://doi.org/10.1093/jac/dkv145
25. Paredes CVT (2009) Actualización en pruebas de susceptibilidad antifúngica. Rev Chil Infectol 26:144–150
26. Pfaller MA, Burmeister L, Bartlett MS, Rinaldi MG (1988) Multicenter evaluation of four methods of yeast inoculum preparation. J Clin Microbiol 26:1437–1441
27. Pfaller MA, Rinaldi MG, Galgiani JN, Bartlett MS, Body BA, Espinel-Ingroff A, Fromtling RA, Hall GS, Hughes CE, Odds FC (1990) Collaborative investigation of variables in susceptibility testing of yeasts. Antimicrob Agents Chemother 34:1648–1654
28. Fromtling RA, Galgiani JN, Pfaller MA, Espinel-Ingroff A, Bartizal KF, Bartlett MS, Body BA, Frey C, Hall G, Roberts GD (1993) Multicenter evaluation of a broth macrodilution antifungal susceptibility test for yeasts. Antimicrob Agents Chemother 37:39–45
29. Espinel-Ingroff A, Kish CW, Kerkering TM et al (1992) Collaborative comparison of broth macrodilution and microdilution antifungal susceptibility tests. J Clin Microbiol 30:3138–3145
30. Barchiesi F, Colombo AL, Mcgough DA, Rinaldi MG (1994) Comparative study of broth macrodilution and microdilution techniques for in vitro antifungal susceptibility testing of yeasts by using the National Committee for Clinical Laboratory Standards proposed standard. J Clin Microbiol 32:2494–2500
31. Pfaller MA, Bale M, Buschelman B, Lancaster M, Espinel-Ingroff A, Rex JH, Rinaldi MG, Cooper CR, McGinnis MR (1995) Quality control guidelines for National Committee for Clinical Laboratory Standards recommended broth macrodilution testing of amphotericin B , fluconazole and flucytosine. J Clin Microbiol 33:1104–1107
32. Barry AL, Pfaller MA, Brown SD, Espinel-Ingroff A, Ghannoum MA, Knapp C, Rennie RP, Rex JH, Rinaldi MG (2000) Quality control limits for broth microdilution susceptibility tests of ten antifungal agents. J Clin Microbiol 38:3457–3459
33. Performance standards for antifungal susceptibility testing of yeasts (2017) CLSI supplement M60. 1 st ed. 1–12. https://clsi.org/media/1895/m60ed1_sample.pdf
34. Johnson EM (2008) Issues in antifungal susceptibility testing. J Antimicrob Chemother 61:13–18. https://doi.org/10.1093/jac/dkm427
35. Lozano-Chiu M, Nelson PW, Lancaster M, Pfaller MA, Rex JH (1997) Lot-to-lot variability of antibiotic medium 3 used for testing susceptibility of *Candida* isolates to amphotericin B. J Clin Microbiol 35:270–272
36. Ghannoum MA, Ibrahim AS, Fu Y, Shafiq MC, Edwards JE Jr, Criddle RS (1992) Susceptibility testing of *Cryptococcus neoformans* : a microdilution technique. J Clin Microbiol 30:2881–2886
37. Pfaller MA, Grant C, Morthland V, Rhine-Chalberg J (1994) Comparative evaluation of alternative methods for broth dilution susceptibility testing of fluconazole against *Candida albicans*. J Clin Microbiol 32:506–509
38. Rodriguez-tudela JL, Martinez-suarez JV (1994) Improved medium for fluconazole susceptibility testing of *Candida albicans*. Antimicrob Agents Chemother 38:45–48
39. Liu W, Zhang X, Liu Z, Luo X (2011) Impact of pH on the antifungal susceptibility of vaginal *Candida albicans*. Int J Gynecol Obstet 114:278–280
40. Zaragoza O, Mesa-Arango AC, Gómez-López A, Bernal-Martínez L, Rodríguez-Tudela JL, Cuenca-Estrella M (2011) Process analysis of variables for standardization of antifungal susceptibility testing of nonfermentative yeasts. Antimicrob Agents Chemother 55:1563–1570
41. Rang HP, Dale MM, Ritter JM (2008) Pharmacology. Elsevier, Rio de Janeiro
42. Murray PR, Rosenthal KS, Pfaller MA (2017) Medical microbiology. Elsevier, Rio de Janeiro

43. Broach JR (2012) Nutritional control of growth and development in yeast. Genetics 192:73–105
44. Young TW (1999) Brewing microbiology. A Chapman & Hall Food Science Book, London
45. Jastrzębowska K, Gabriel I (2015) Inhibitors of amino acids biosynthesis as antifungal agents. Amino Acids 47:227–249
46. Lee KL, Buckley HR, Campbell CC (1975) An amino acid liquid synthetic medium for the development of mycelial and yeas forms of *Candida albicans*. Sabouraudia 13:148–153
47. McCoy TA, Maxwell M, KruseJr PF (1959) Amino acid requirements of the Novikoff hepatoma in vitro. Exp Biol Med 100:115–118
48. Moore GE, Mount D, Tara G, Schwartz N (1963) Growth of human tumor cells in suspension cultures. AACR J Arch 23:1735–1741
49. Radetsky M, Wheeler RC, Roe MH, Todd JK (1986) Microtiter broth dilution method for yeast susceptibility testing with validation by clinical outcome. J Clin Microbiol 24:600–606
50. Rex JH, Cooper CR, Merz WG et al (1995) Detection of amphotericin B-resistant *Candida* isolates in a broth-based system. Antimicrob Agents Chemother 39:906–909
51. Cuenca-Estrella M, Díaz-Guerra TM, Mellado E, Rodríguez-Tudela JL (2001) Detection of resistance to amphotericin B in *Candida* isolates by using Iso-Sensitest broth. Antimicrob Agents Chemother 45:2070–2074
52. Cruz RC, Werneck SMC, Oliveira CS et al (2013) Influence of different media, incubation times, and temperatures for determining the MICs of seven antifungal agents against *Paracoccidioides brasiliensis* by microdilution. J Clin Microbiol 51:436–443
53. Goughenour KD, Balada-Llasat JM, Rappleye CA (2015) Quantitative microplate-based growth assay for determination of antifungal susceptibility of *Histoplasma capsulatum* yeasts. J Clin Microbiol 53:3286–3295
54. Peano A, Pasquetti M, Tizzani P, Chiavassa E, Guillot J, Johnson E (2017) Methodological issues in antifungal susceptibility testing of *Malassezia pachydermatis*. J.Fungi 3:2–15. https://doi.org/10.3390/jof3030037
55. Ferreira CMH, PintoISS SEV, SoaresHMVM (2015) (un)suitability of the use of pH buffers in biological, biochemical and environmental studies and their interaction with metal ions-a review. RSC Adv 5:30989–31003
56. Will MA, Clark NA, Swain JE (2011) Biological pH buffers in IVF: help or hindrance to success. J Assist Reprod Genet 28:711–724
57. Gadea I, Cuenca M, Gegúndez MI et al (1997) Effect of pH and buffer system on the in-vitro activity of five antifungals against yeasts. J Antimicrob Chemother 39:453–459. https://doi.org/10.1093/jac/39.4.453
58. McIntyre KA, Galgiani JN (1989) pH and other effects of the antifungal activity of cilofungin (LY121019). Antimicrob Agents Chemother 33:731–735
59. Arthington-Skaggs BA, Warnock DW, Morrison CJ (2000) Quantitation of *Candida albicans* ergosterol content improves the correlation between in vitro antifungal susceptibility test results and in vivo outcome after fluconazole treatment in a murine model of invasive candidiasis. Antimicrob Agents Chemother 44:2081–2085
60. Ostrosky-Zeichner L, Rex JH, Pfaller MA, Diekema DJ, Alexander BD, Andes D, Brown SD, Chaturvedi V, Ghannoum MA, Knapp CC, Sheehan DJ, Walsh TJ (2008) Rationale for reading fluconazole MICs at 24 hours rather than 48 hours when testing *Candida* spp. by the CLSI M27-A2 standard method. Antimicrob Agents Chemother 52:4175–4177. https://doi.org/10.1128/AAC.00420-08
61. Kaya E, Özbilge H (2012) Determination of the eff ect of fluconazole against *Candida albicans* and *Candida glabrata* by using microbroth kinetic assay. Turk J Med Sci 42:325–328
62. Berkow EL, Lockhart SR, Ostrosky-Zeichner L (2020) Antifungal susceptibility testing: current approaches. Clin Microbiol Rev 33:1–30
63. Balouiri M, Sadiki M, Ibnsouda SK (2016) Methods for in vitro evaluating antimicrobial activity: a review. J Pharm Anal 1:671–679. https://doi.org/10.1016/j.jpha.2015.11.005
64. Kuhn DM, Balkis M, Chandra J, Mukherjee PK, Ghannoum MA (2003) Uses and limitations of the XTT assay in studies of *Candida* growth and metabolism. J Clin Microbiol 41:506–508
65. Stoppa MA, Casemiro LA, Vinholis AHC, Cunha WR, Silva MLA, Martins CHG, Furtado NAJC (2009) Comparative study between the CLSI hair and EUCAST hair methodologies recommended for antifungal activity evaluation. Quim Nova 32:498–502
66. Arikan S (2007) Current status of antifungal susceptibility testing methods. Med Mycol 45:569–587. https://doi.org/10.1080/13693780701436794
67. Liu M, Seidel V, Katerere DR, Gray AI (2007) Colorimetric broth microdilution method for the antifungal screening of plant extracts against yeasts. Methods 42:325–329. https://doi.org/10.1016/j.ymeth.2007.02.013
68. Monteiro MC, Cruz ML, Cantizani J et al (2012) A new approach to drug discovery: high-throughput screening of microbial natural extracts against *Aspergillus fumigatus* using resazurin. J Biomol Screen 17:542–549. https://doi.org/10.1177/1087057111433459