Multicenter evaluation of the reproducibility of the proposed antifungal susceptibility testing method for fermentative yeasts of the Antifungal Susceptibility Testing Subcommittee of the European Committee on Antimicrobial Susceptibility Testing (AFST-EUCAST)

M. Cuenca-Estrella[1], C. B. Moore[2], F. Barchiesi[3], J. Bille[4], E. Chryssanthou[5], D. W. Denning[2], J. P. Donnelly[6], F. Dromer[7], B. Dupont[7], J. H. Rex[8], M. D. Richardson[9], B. Sancak[8], P. E. Verweij[6], J. L. Rodríguez-Tudela[1] and the AFST Subcommittee of the European Committee on Antimicrobial Susceptibility Testing

[1]Instituto de Salud Carlos III, Majadahonda, Spain, [2]University of Manchester, Manchester, UK, [3]Istituto di Malattie Infettive e Medicina Pubblica, Ancona, Italy, [4]Center Hospitalier Universitaire Vaudois, Lausanne, Switzerland, [5]Karolinska Hospital, Stockholm, Sweden, [6]University Medical Center Nijmegen, Nijmegen, The Netherlands, [7]Institute Pasteur, Paris, France, [8]University of Texas Medical School, Houston, Texas, USA and [9]University of Helsinki, Helsinki, Finland

Objective To evaluate the intra- and inter-laboratory reproducibility of a new standard for susceptibility testing of fermentative yeasts. This standard is based on the M27-A procedure of the National Committee for Clinical Laboratory Standards (NCCLS), but incorporates several modifications, including spectrophotometric growth-dependent endpoint reading.

Methods Nine laboratories participated in the study. Common material lots were used to test six *Candida* species (one each of *C. albicans*, *C. tropicalis*, *C. parapsilosis*, *C. glabrata*, *C. krusei*, and *C. lusitaniae*), and two quality control strains (*C. krusei* ATCC6258 and *C. parapsilosis* ATCC22019). Triplicate testing on three separate days was performed in microtiter format with RPMI−2% glucose, pH 7.0. Flucytosine, fluconazole and itraconazole were tested. In total, 3888 MIC values were included in the analyses. Reproducibility was calculated by means of agreement (percentage of MICs within one two-fold dilution of the mode) and intraclass correlation coefficient (ICC, maximum value of 1).

Results The average intra-laboratory agreements were 99% and 96% after 24 h and 48 h of incubation, respectively, with ICCs of 0.98 and 0.97 ($P < 0.05$). Two strains exhibiting a trailing effect showed intra-laboratory agreement of 92% and ICCs of <0.91 at 48 h. The inter-laboratory agreement was 94% and 88% after 24 h and 48 h, respectively, with ICCs of 0.93 and 0.91 ($P < 0.05$). Lower values of agreement and ICCs were obtained for strains exhibiting trailing after 48 h of incubation. Itraconazole yielded the lowest values of reproducibility.

Conclusion The new procedure of EUCAST for antifungal susceptibility testing is a reproducible method within and between laboratories and offers several advantages over the NCCLS approved method.

Keywords AFST-EUCAST, antifungal susceptibility testing, NCCLS–EUCAST agreement

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Corresponding author and reprint requests: J. L. Rodríguez-Tudela, Servicio de Micología, Centro Nacional de Microbiología, Instituto de Salud Carlos III, Ctra. de Majadahonda a Pozuelo Km. 2. 28220 Majadahonda, Spain
Tel: +34 91 5097961
Fax: +34 91 5097966
E-mail: juanl.rodriguez-tudela@isciii.es

INTRODUCTION

The National Committee for Clinical Laboratory Standards (NCCLS) Subcommittee on Antifungal Susceptibility has standardized the in vitro susceptibility testing of fungi. It has published an

approved standard for testing of *Candida* spp. and *Cryptococcus neoformans* (document M27-A), and has proposed guidelines for conidia-forming fungi (document M38-P) [1,2]. These reference methodologies represent a new milestone in the evolution of medical mycology. The techniques are characterized by their high reliability and reproducibility, which are essential features for identifying organisms unlikely to respond to particular antifungal treatments [3]. However, the utilization of NCCLS procedures still has some limitations: the trailing phenomenon with azoles, unreliable detection of resistance to amphotericin B, and poor growth of some organisms resulting in extended turnaround time. Despite these limitations, the NCCLS methodology has become the reference procedure, and any progress in this field must take this procedure into account [4].

The Antifungal Susceptibility Testing Subcommittee of the European Committee on Antibiotic Susceptibility Testing (AFST-EUCAST) has proposed an alternative standard for the determination of MICs for fermentative species of yeasts by broth microdilution methodology [5]. The proposed standard of the AFST-EUCAST is based on the reference NCCLS M27-A procedures [1], but incorporates some modifications in order to obtain an objective MIC result, allow for automation of the system, and shorten the incubation period for MIC determination (24 h). The modifications include RPMI-1640 supplemented with 2% dextrose as assay medium, an inoculum size of 0.5×10^5 to 2.5×10^5 CFU/mL, flat-bottomed trays, spectrophotometric reading, and a 50% inhibition endpoint for azole agents and flucytosine. In addition, sufficient growth has to have occurred (measured spectrophotometrically) for an endpoint to be read. Previous work has suggested that a large inoculum size and glucose supplementation of assay medium for AFST may falsely elevate the MICs for yeasts [6], but recent reports have pointed out that these modifications do not affect the reproducibility, and high agreement with the NCCLS reference procedure was obtained [4,7]. Since several studies have confirmed that the modified M27-A technique with 2% glucose has the advantage of reducing the incubation time necessary to obtain sufficient growth to determine the MIC [8,9], supplementation with 2% glucose was included in the proposed standard for antifungal susceptibility testing of fermentative yeasts of the AFST-EUCAST. Species of *Cryptococcus and* other yeasts such as *Trichosporon* and *Rhodotorula* have not been included in the proposed standard. Non-fermentative yeasts grow poorly in the assay medium currently recommended for reference susceptibility testing procedures. A recent report has pointed out that cultivation in culture medium other than RPMI with constant agitation could be an alternative for performing susceptibility testing of *Cryptococcus* spp. [10]. The AFST-EUCAST is planning collaborative studies in order to propose a standard for antifungal susceptibility testing of non-fermentative yeasts.

After preliminary evaluation of the agreement of the proposed standard with the reference procedure of the NCCLS was completed, cooperative multicenter collaboration was needed to ascertain the reproducibility of the EUCAST technique. In order to assess the intra- and inter-laboratory reproducibility of the AFST-EUCAST proposed standard for fermentative yeasts, a multicenter exercise was performed. This involved testing a panel of clinical isolates of different yeast species, including quality control strains, with flucytosine, fluconazole, and itraconazole. The details of this study and its implications form the subject of this work.

METHODS

Participants

Nine laboratories took part in the exercise: (1) Servicio de Micología, Centro Nacional de Microbiología, Instituto de Salud Carlos III, Majadahonda, Spain; (2) Departments of Microbiology and Medicine, Hope Hospital, Salford, UK; (3) Istituto di Malattie Infettive e Medicina Pubblica, Universita degli Studi di Ancona, Ancona, Italy; (4) Institut de Microbiologie, Center Hospitalier Universitaire Vaudois, Lausanne, Switzerland; (5) Department of Clinical Microbiology, Karolinska Hospital, Stockholm, Sweden; (6) Department of Medical Microbiology, University Medical Center Nijmegen, Nijmegen, The Netherlands; (7) Unite de Mycologie, Institute Pasteur, Paris, France; (8) Division of Infectious Diseases, Center for the Study of Emerging and Reemerging Pathogens, University of Texas Medical School, Houston, Texas, USA; and (9) Department of Bacteriology and Immunology, Haartman Institute, University of Helsinki, Helsinki, Finland.

Table 1 Percentages of intra-laboratory agreement for each drug–strain combination

	Percentages of agreement by antifungal agent/incubation time							
	Flucytosine		Fluconazole		Itraconazole		Means	
Strain	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h
Candida krusei ATCC6258	100.0	100.0	100.0	100.0	100.0	98.6	100.0	99.5
Candida krusei strain 16[a]	100.0	100.0	100.0	100.0	100.0	98.6	100.0	99.5
Candida tropicalis strain 18	100.0	95.2	98.4	95.2	100.0	90.4	99.4	93.6
Candida albicans strain 19	100.0	100.0	100.0	100.0	96.8	93.6	98.9	97.8
Candida parapsilosis strain 24	100.0	100.0	100.0	90.4	100.0	95.2	100.0	95.2
Candida lusitaniae strain 27	100.0	100.0	100.0	95.2	98.4	92.0	99.4	95.7
Candida glabrata strain 31	100.0	100.0	96.8	93.6	92.0	80.5	96.2	92.4
Candida parapsilosis ATCC 22019	100.0	100.0	100.0	100.0	100–0	96.8	100.0	98.9
Means	100.0	99.4	99.4	96.8	98.4	93.2	99.2	96.4

[a]Strains belong to a panel of 60 clinical isolates used by the AFST-EUCAST for standardization exercises. Participants received six encoded isolates and two ATCC organisms. Participants did not know the identity of the six encoded strains.

Fungi

Every participant tested a panel of six encoded *Candida* spp. strains. Table 1 shows the codes and identification of isolates employed in the study. These strains were selected because they had exhibited on-scale MIC values of agents tested in preliminary studies [4]. *Candida parapsilosis* ATCC22019 and *Candida krusei* ATCC6258 were incorporated as quality control strains in each set of experiments [1]. Yeast stock cultures were stored at −70 °C.

Assay media

The assay medium was RPMI-1640 without sodium bicarbonate and with L-glutamine buffered to pH 7.0 with 0.165 M morpholinepropanesulfonic acid and supplemented with 18 g of glucose per liter to reach a final concentration of 2% (RPMI−2% glucose). Culture medium was prepared as a double-strength solution and sterilized by filtration. A common lot was used throughout the experiments (lot no. 17359, Oxoid SA, Madrid, Spain).

Antifungal agents

The antifungal agents used in this study were: flucytosine (lot no. 805007, Hoffman La Roche, Basel, Switzerland), fluconazole (lot no. 86386-73-4, Pfizer Ltd, Sandwich, UK), and itraconazole (batch no. PL4401, Janssen Pharmaceutica, Beerse, Belgium). Antifungal agents were obtained from a single lot directly from their respective manufacturers as reagent-grade powders. The potency of each drug was 100%. The powders were stored as recommended by the manufacturers, and aliquots (100 mg) of these antifungal agents were sent to each participant. Stock solutions were prepared in dimethylsulfoxide (DMSO), except for flucytosine, which was dissolved in sterile distilled water. Stock solutions were made up at concentrations of 100 times the highest concentration to be tested, and were frozen in aliquots at −70 °C until used.

Preparation of inoculum

The yeast isolates were grown on Sabouraud dextrose agar for 24 h at 35 °C. Suspensions were prepared by picking five distinct colonies of $\geq$1 mm diameter and suspending them in 5 mL of distilled sterile water. A spectrophotometric procedure for inoculum preparation was used [1]. The final inoculum suspension contained between 0.5×10^5 and 2.5×10^5 CFU/mL.

Susceptibility testing

Triplicate testing on three separate days was performed. The methodology used strictly followed the proposed standard of the EUCAST for determination of MIC by broth dilution of fermentative species of yeasts [5]. Sterile plastic microtiter plates containing flat-bottomed wells were utilized (Corning Costar Europe, Badhoevedorp, The Netherlands). Each participant prepared their own trays. The plates contained 100 μL of two-fold serial dilutions of the antifungal drugs per well. Two drug-free medium wells for sterility and

growth controls were employed. Trays were inoculated with 100 μL of final inoculum per well. Final concentrations of drugs were 0.12–64.0 mg/L for flucytosine and fluconazole, and 0.015–8.0 mg/L for itraconazole The microdilution plates were incubated at 35 °C for 48 h. Stationary cultures were performed. The MICs were determined spectrophotometrically after 24 and 48 h of incubation.

Endpoint determination

Laboratories determined the endpoint with a spectrophotometer at a wavelength of 450 nm. The MIC was defined as the lowest drug concentration resulting in a reduction of growth of 50% or more compared with the growth of the control. An OD of $\leq$0.5 after 24 h of incubation indicated poor growth, and plates were reincubated for a further 12–24 h and then read. Failure to reach an OD of 0.5 after 48 h constituted a failed test.

Statistical analysis

Intra- and inter-laboratory reproducibility of the results for each drug–strain combination was evaluated by agreement between MIC results. Intra-laboratory agreement was calculated by employing the nine MIC values obtained per participant for each drug–strain combination. Inter-laboratory agreement was evaluated by concordance between MIC values determined by different laboratories. The agreement was defined as percentage of MICs within one two-fold dilution interval of the mode. A level of 85% agreement was selected to validate the exercise. In addition, intraclass correlation coefficients (ICCs) were calculated. These coefficients compared the results of the MIC determinations transformed on $\log_2$ data of the eight strains. A two-way random effect model was utilized to calculate the ICCs with a confidence interval of 95% (CI 95%). The ICC was calculated using the formula ICC = (group mean square − error mean square)/(group mean square + error mean square), and thus has a maximum value of 1 if there is a perfect correlation and a minimum value of −1 if there is complete absence of correlation. A P-value <0.05 was considered significant. Both on-scale and off-scale results were included in the analysis. All statistical analysis was done with the Statistical Package for the Social Sciences (SPSS, version 10.0) (SPSS SL, Madrid, Spain).

RESULTS

Intra-laboratory agreement

The percentages of agreement were calculated per participant and for each drug–strain combination. Overall, the intra-laboratory agreement was very high. After 24 h of incubation, the values of agreement ranged between 92.0% and 100% (average agreement of 99.2%), and after 48 h they ranged between 80.5% and 100% (average agreement of 96.4%). Table 1 shows the percentages of agreement obtained for each drug–strain combination. The reproducibility of MIC values of each laboratory was also high. The mean ICC obtained per participant after 24 h of incubation was 0.98 (95% CI = 0.97–0.99), and that after 48 h was 0.97 (95% CI = 0.96–0.98) ($P < 0.05$). Per strain tested, the lowest values of reproducibility were for isolates of *Candida tropicalis and Candida glabrata* after 48 h of incubation (ICCs of $<$0.90 and $<$0.91, respectively). Per antifungal agent, the worst reproducibility was obtained for itraconazole after 48 h of incubation (average ICC per participant of 0.94 (95% CI = 0.91–0.96)).

Inter-laboratory agreement

Table 2 shows the percentages of inter-laboratory agreement by antifungal agent–strain for MIC values obtained after 24 and 48 h of incubation. Agreements among MIC values from participants were very high, with average percentages of 94.1% after 24 h of incubation and 87.8% after 48 h. When each organism was analyzed, after 24 h of incubation the lowest percentage of agreement was obtained for *Candida glabrata* (89.5%). However, after 48 h, several isolates exhibited percentages of agreement below 85%. When each antifungal agent was analyzed, itraconazole showed the lowest average percentages of inter-laboratory agreement, particularly for results obtained after 48 h of incubation (76.6%). Table 3 shows ICCs by antifungal agent–strain for MIC values obtained after 24 h and 48 h of incubation. The mean ICCs were 0.93 (95% CI = 0.91–0.98) and 0.91 (95% CI = 0.88–0.95) after 24 h and 48 h of incubation, respectively. These ICCs indicate a very high correlation among MIC results from each participant ($P < 0.05$). No significant differences were observed when each organism was analyzed. However, for antifungal agents, itraconazole exhibited average ICCs of 0.90 and 0.89 after 24 h and 48 h of incubation, respectively.

Table 2 Percentages of inter-laboratory agreement for each drug–strain combination

	Percentages of agreement by antifungal agent/incubation time							
	Flucytosine		Fluconazole		Itraconazole		Means	
Strain	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h
Candida krusei ATCC 6258	98.1	100.0	100.0	100.0	90.5	83.3	96.2	94.4
Candida krusei strain 16[a]	100.0	100.0	100.0	100.0	85.6	70.3	95.2	90.1
Candida tropicalis strain 18	100.0	100.0	98.1	50.0	87.0	75.9	95.0	75.3
Candida albicans strain 19	100.0	100.0	85.2	94.4	85.9	62.9	90.4	85.8
Candida parapsilosis strain 24	100.0	100.0	100.0	75.9	92.6	70.3	97.5	82.1
Candida lusitaniae strain 27	96.3	100.0	100.0	100.0	85.3	98.1	93.9	99.4
Candida glabrata strain 31	100.0	100.0	83.3	75.9	85.3	70.3	89.5	82.0
Candida parapsilosis ATCC 22019	100.0	100.0	100.0	100.0	86.3	81.5	95.4	93.8
Means	99.3	100.0	95.8	86.9	87.3	76.6	94.1	87.8

[a]Strains belong to a panel of 60 clinical isolates used by the AFST-EUCAST for standardization exercises. Participants received six encoded isolates and two ATCC organisms. Participants did not know the identity of the six encoded strains.

Table 3 Correlation expressed in ICCs among MIC values for each drug–strain combination

	Percentages of agreement by antifungal agent/incubation time							
	Flucytosine		Fluconazole		Itraconazole		Means	
Strain	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h
Candida krusei ATCC 6258	0.99	0.97	0.98	0.95	0.95	0.94	0.97	0.95
Candida krusei strain 16[a]	0.98	0.96	0.94	0.92	0.90	0.90	0.94	0.93
Candida tropicalis strain 18	0.94	0.91	0.94	0.90	0.89	0.88	0.92	0.90
Candida albicans strain 19	0.96	0.95	0.92	0.91	0.90	0.89	0.93	0.92
Candida parapsilosis strain 24	0.95	0.95	0.92	0.89	0.88	0.88	0.92	0.91
Candida lusitaniae strain 27	0.95	0.92	0.91	0.90	0.91	0.91	0.92	0.91
Candida glabrata strain 31	0.96	0.93	0.90	0.89	0.88	0.87	0.91	0.90
Candida parapsilosis ATCC 22019	0.99	0.94	0.96	0.94	0.92	0.91	0.96	0.93
Means	0.96	0.94	0.93	0.91	0.90	0.89	0.93	0.91

[a]Strains belong to a panel of 60 clinical isolates used by the AFST-EUCAST for standardization exercises. Participants received six encoded isolates and two ATCC organisms. Participants did not know the identity of the six encoded strains.

Antifungal susceptibility results of quality control strains

The MIC values obtained for the control organisms varied by no more than three two-fold dilutions. For *Candida krusei* ATCC 6258, MICs of flucytosine ranged between 1 and 4 mg/L, those of fluconazole ranged between 8 and 32 mg/L, and those of itraconazole ranged between 0.06 and 0.25 mg/L. For *Candida parapsilosis* ATCC 22019, MICs of flucytosine ranged between 0.12 and 0.50 mg/L, those of fluconazole ranged between 1 and 4 mg/L, and those of itraconazole ranged between 0.03 and 0.12 mg/L.

DISCUSSION

The NCCLS reference method for susceptibility testing of yeasts includes some alternative media for special circumstances, but indicates that the utility of these modifications remains to be established [1]. One alternative medium is RPMI supplemented with glucose at a final concentration of 20 g/L. It has been pointed out that glucose supplementation aids the growth of yeasts and may simplify endpoint determination, because of higher turbidity of the growth control [8,9]. In addition, there is an increase in growth when higher inoculum sizes are employed (10^4 or 10^5 CFU/mL).

The combination of higher inoculum sizes and assay medium supplemented with glucose allows the incubation time to be shortened from 48 to 24 h [4]. In contrast, however, other reports have indicated that a large inoculum size and glucose supplementation may falsely elevate the MICs, mainly at 48 h, and that glucose supplementation does not stimulate heavier growth after 24 h of incubation [6].

With regard to endpoint determination, the NCCLS reference method of visually grading turbidity can become a subjective and difficult task, particularly with fungistatic agents. The trailing phenomenon makes visual determination of azole MICs problematic, because of partial inhibition of fungal growth. An alternative to the reference procedure is the determination of the endpoint by automated spectrophotometric methods [9,11]. Microtiter trays and automated reading allow the determination of endpoints at different levels of growth inhibition and are free from subjectivity. Several studies have shown, however, that MIC values obtained by spectrophotometric reading exhibit poor agreement with results achieved by visual reading, particularly for azole susceptibility testing after 24 h of incubation [11]. Other findings have indicated that the addition of 2% glucose and a larger inoculum size improve the agreement between the results of the spectrophotometric method and the reference procedures [4].

In order to address these issues, the AFST-EUCAST carried out preliminary work to compare M27-A procedures with a new method for the determination of MICs for fermentative species of yeasts by broth microdilution methodology. These studies evaluated the combined influence of assay medium, inoculum size and reading method on results of susceptibility testing [4,7]. As a result, the AFST-EUCAST has proposed a standard micromethod for MIC determination for glucose fermenter yeasts. This micro-method yielded a reproducible technique that showed better agreement with NCCLS reference procedures and shortened the incubation period necessary to obtain reliable MIC values. In addition, spectrophotometric reading offered an advantage over the visual method by providing a more objective and automated MIC determination [12].

After the proposal of this standard, a collaborative study was mandatory to ascertain the reproducibility of the technique. Herein we present the results of the multicenter evaluation of the reproducibility of the proposed AFST-EUCAST standard. Percentages of intra- and inter-laboratory agreements, and reproducibility and correlation indices, were very high, particularly for MICs obtained after 24 h of incubation. A level of 85% agreement or greater had been selected to validate the exercise. Percentages of agreement were higher than this for MICs obtained at both 24 and 48 h. In addition, reproducibility and correlation indices were statistically significant ($P < 0.05$). Despite the high average agreement, some strains included in the exercise exhibited lower percentages of agreement at 48 h, particularly *Candida tropicalis and Candida glabrata* isolates. These strains were all subject to such significant trailing growth that the MICs after 24 h were much lower than after 48 h. It could be argued that trailing growth can be an important source of variability and inaccuracy in MIC determination after 48 h of incubation. Some may argue that, with the trailing strains, we do not know what the 'correct' MIC is, and that in vitro–in vivo correlation work would have to be done to find whether the 'true' MIC value is that of 24 h or that of 48 h. Increasingly, however, work suggests that trailing growth represents an artifact and that the lower MIC is the correct one [13–15].

Another point to consider is the reproducibility of the MICs of itraconazole, which were lower than those of flucytosine and fluconazole. Itraconazole is a lipophilic drug insoluble in water, and must be diluted in an appropriate non-aqueous solvent such as DMSO. For preparing working solutions, susceptibility testing standards recommend series of dilutions from the antifungal stock solution in the same solvent in order to avoid dilution artifacts that result from precipitation of compound [1,2]. Nevertheless, obtaining homogeneous solutions of itraconazole is not straightforward, and can have a significant effect on the reproducibility of MIC results. Moreover, a recent report confirmed the relative influence of different test variables on the itraconazole MICs and that each fungal isolate can respond differently to combinations of process variables in the test [16].

Amphotericin B has not been included in the AFST-EUCAST proposed standard. Susceptibility testing using RPMI as the assay medium is not able to detect resistance to amphotericin B in yeasts. RPMI medium yields a range of amphotericin B MICs, spanning only three or four two-fold dilutions. This narrow range precludes reliable discri-

mination between susceptible and resistant isolates [3]. The AFST-EUCAST is planning additional collaborative studies to standardize reliable methods for detecting resistance to amphotericin B. In any case, reports have indicated that MICs of amphotericin B obtained by AFST-EUCAST methodology exhibit high percentages of agreement with those obtained by the NCCLS reference technique [12].

In conclusion, the AFST-EUCAST proposed standard is a reproducible and reliable technique for testing the susceptibility of fermentative species of yeasts within and between laboratories. Reproducibility is better for MIC values obtained at 24 h than for those obtained at 48 h, due to strains exhibiting trailing growth.

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