



Narrative review

How to: perform antifungal susceptibility testing of microconidia-forming dermatophytes following the new reference EUCAST method E.Def 11.0, exemplified by *Trichophyton*[☆]

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ABSTRACT

Background: Antifungal drug resistance in dermatophytes was first reported shortly after the turn of the millennium and has today been reported in *Trichophyton* and occasionally in *Microsporum*, but not in *Epidermophyton* species. Although drug resistance in dermatophytes is not routinely investigated, resistance in *Trichophyton* spp. is increasingly reported worldwide. The highest rates are observed in India (36% and 68% for terbinafine (MIC ≥ 4 mg/L) and fluconazole (MICs ≥ 16 mg/L), respectively), and apparently involve the spread of a unique clade related to the *Trichophyton mentagrophytes/Trichophyton interdigitale* complex.

Objectives: The European Committee on Antimicrobial Susceptibility Testing Subcommittee on Antifungal Susceptibility Testing (EUCAST-AFST) has released a new method (E.Def 11.0) for antifungal susceptibility testing against microconidia-forming dermatophytes including tentative MIC ranges for quality control strains and tentative breakpoints against *Trichophyton rubrum* and *T. interdigitale*. Here, the details of the new procedure E.Def 11.0 are described.

Sources: This technical note is based on the multicentre validation of the EUCAST dermatophyte antifungal susceptibility testing method, the mould testing method (E.Def 9.3.2) and the updated quality control tables for antifungal susceptibility testing document, v 5.0 (available on the EUCAST website).

Contents: The method is based on the EUCAST microdilution method for moulds but significant differences include: (a) an altered test medium selective for dermatophytes; (b) an altered incubation time and temperature; and (c) a different end-point criterion (spectrophotometric determination) of fungal growth. It can easily be implemented in laboratories already performing EUCAST microdilution methods and has been validated for terbinafine, voriconazole, itraconazole and amorolfine against *T. rubrum* and *T. interdigitale*.

Implications: This standardized procedure with automated end-point reading will allow broader implementation of susceptibility testing of dermatophytes and so facilitate earlier appropriate therapy.

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This is important, as resistance is rapidly emerging and largely underdiagnosed. **Maiken C. Arendrup, Clin Microbiol Infect 2021;27:55**

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Introduction

Antifungal susceptibility tests are performed for fungi causing disease, especially when infections are invasive, relapsing or failing therapy, when inherent or acquired resistance is a possibility, or when susceptibility cannot reliably be predicted from the species identification alone. Antifungal susceptibility testing (AFST) is also important in resistance surveillance, in epidemiological studies and for comparison of the *in vitro* activity of new and existing agents.

Dilution methods are used to establish the MICs of antimicrobial agents. These are the reference methods for antimicrobial susceptibility testing and are mainly used: to (a) establish the activity of new antimicrobial agents; (b) confirm the susceptibility of organisms that give equivocal results in other test formats (such as commercial susceptibility tests); and (c) determine the susceptibility of organisms where other test formats may be unreliable or not yet validated (which is still a common scenario for susceptibility testing of fungi and dermatophytes in particular). In dilution tests, fungi are tested for their ability to produce sufficient growth in microplate wells of broth culture medium containing serial dilutions of the antimicrobial agents (broth microdilution).

The antifungal MIC is defined as the lowest concentration, in mg/L, of an agent that inhibits the growth of a fungus. The MIC informs about the susceptibility or resistance of the organism to the antifungal agent, which can help in treatment decisions.

Dermatophyte infections are rarely invasive but when they involve the scalp or nails, systemic therapy for weeks to months is required [1,2]. Systemic antifungal therapy is indicated also in cutaneous dermatophytosis presenting with extensive lesions or in patients with lack of response to topical therapy [3,4]. Presumptive diagnosis often relies on clinical findings and direct microscopic examination only, but differential diagnoses are many, systemic therapy is associated with risk of side effects, and resistance in

Trichophyton species is rapidly rising [5–16]. Hence, mycological diagnosis and standardized AFST methods for determining the *in vitro* susceptibilities of clinical isolates of dermatophytes are needed to limit inappropriate therapy, unnecessary toxicity and selection of resistance [17].

Dermatophytes grow slowly and often require 4–7 days of culture or more. Skin scrapings and nail samples are not sterile and although cultured on selective agars, MIC determination in non-selective broth medium is often challenged by contamination (Fig. 1). Such contamination interferes with end-point reading and often necessitates repetition of the test after isolation, which adds days or even weeks to the turnover time before a result can be provided. For these reasons, European Committee on Antimicrobial Susceptibility Testing (EUCAST) initially investigated if addition of cycloheximide and chloramphenicol to the standard EUCAST AFST growth medium, to render it selective for dermatophytes, interfered with microdilution MIC determination of selected wild-type and mutant *Trichophyton* isolates. Subsequently, EUCAST investigated the optimal test conditions for a dermatophyte EUCAST microdilution method, including various end-point definitions for the correct separation of susceptible wild-type and resistant non-wild-type clinical isolates of *Trichophyton rubrum* and *Trichophyton interdigitale* in a multicentre study [18].

This first version of the standard is based on the general EUCAST principles for microtitre plate production described in the yeast and mould microdilution method documents (E.Def 7.3.2 and E.Def 9.3.2) with subsequent addition of cycloheximide and chloramphenicol during the inoculation step. This will allow the use of plates already prepared for mould testing (and thus prepared without cycloheximide and chloramphenicol in the plates) rather than requiring production of special plates for dermatophyte testing. Details on plate production are given in the Supplementary material (Appendix S1).

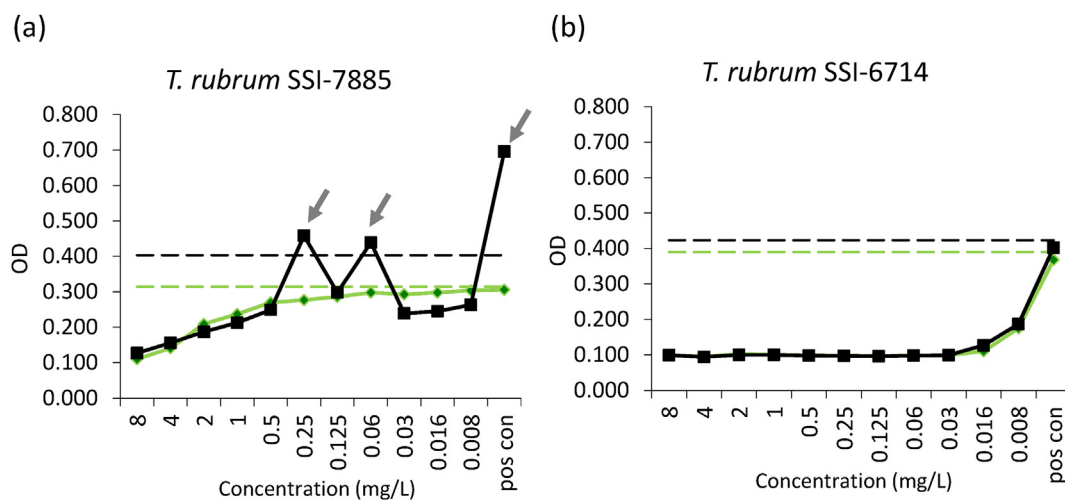


Fig. 1. Terbinafine growth inhibition curves for (a) a resistant *Trichophyton rubrum* isolate (SSI-7885) harbouring a F397L target gene alteration and (b) a susceptible wild-type *T. rubrum* isolate (SSI-6714) tested following the E.Def 9.3.2 mould testing method (black curve) and the new E.Def 11.0 dermatophyte testing method with cycloheximide and chloramphenicol-supplemented medium (green curve). Solid lines indicate the inhibition curves, dashed lines the mean of four positive growth controls. Contaminated wells interfering with the susceptibility testing of the resistant isolate with the E.Def 9.3.2 method are indicated with grey arrows. The background is not subtracted (–OD 0.100).

Scope

This EUCAST standard describes a suitable method for testing the susceptibility of microconidia-producing dermatophytes to antifungal agents by determination of the MIC. MICs show the *in vitro* activity of a given antifungal drug under the test conditions described, and can be used for patient management in conjunction with other factors, such as pharmacokinetics, pharmacodynamics and resistance mechanisms. The MIC permits microorganisms to be categorized as Susceptible (S), susceptible, Increased exposure (I), or Resistant (R) to an antifungal drug when appropriate break-points are applied [19]. In addition, MIC distributions can be used to define wild-type or non-wild-type fungal populations when species-specific epidemiological cut-off values (ECOFFs) are applied.

The method described herein is intended to provide a suitable, easy and economic method for testing the susceptibility to antifungal agents against *Trichophyton* spp. and to facilitate an acceptable degree of conformity, e.g. agreement within specified ranges, between laboratories. Many factors influence the MIC of filamentous fungi against antifungal agents as shown by Rambali et al. [20]. For example the MIC of itraconazole against *Aspergillus* was profoundly influenced by shape of the microdilution well, inoculum concentration, temperature and length of incubation time. As technical laboratory factors are of utmost importance, this standard focuses on testing conditions including inoculum preparation and inoculum size, incubation time and temperature, and end-point definition. The terms and definitions used as basis for this procedure are found in the Supplementary material (Appendix S1).

Test procedures

The general test procedures concerning type of microtitre plates, choice of medium, preparation of stock and working solutions of antimicrobial agents, and preparation and storage of prepared plates with antifungals are identical to the recommendations in the mould (E.Def 9.3.2) reference method document, available at <https://www.eucast.org/astoffungi/>. The details are found in the Supplementary material (Appendix S1) and Tables 1 and 2.

General

The test is performed in flat-bottom well microdilution plates. Different plastics are likely to impact on drug binding, which may affect MIC values. The validation of this method has been performed using tissue-treated microdilution plates, and the use of such plates is therefore more likely to yield similar MIC values. The method is based on the preparation of antifungal agent working solutions in 100- μ L volumes per well to which 100 μ L of inoculum supplemented with cycloheximide and chloramphenicol is added.

Cycloheximide and chloramphenicol working solutions are prepared in the following concentrations: cycloheximide: 100 mg/mL (= 100 000 mg/L) in dimethylsulphoxide, followed by filtration (0.2 mm) (available ready to use from Sigma-Aldrich, St Louis, MO,

USA; Cat. No. C4859). Chloramphenicol: 50 mg/mL (= 50 000 mg/L) in ethanol.

Solutions must be prepared taking into account the potency of the lot of antifungal drug powder that is being used. The amount of powder or diluent required to prepare a standard solution may be calculated as described in Table 1.

Preparation of inoculum supplemented with cycloheximide and chloramphenicol

Standardization of the inoculum is essential for accurate and reproducible antifungal susceptibility tests. The final inoculum must be between 1×10^5 CFU/mL and 2.5×10^5 CFU/mL.

Microconidia suspension method

The isolates are subcultured on Sabouraud dextrose, potato dextrose agar or malt agar supplemented with cycloheximide (300 mg/L) and chloramphenicol (50 mg/L) and incubated at 25°C–28°C for 4–7 days. It may be advisable to inoculate two or three agar plates per isolate to ensure that a sufficient amount of microconidia can be harvested. Other culture media selective for dermatophyte growth, and where the fungus is able to sporulate sufficiently, can be used. Inoculum suspensions are prepared from fresh, mature cultures. In some cases, an extended incubation is required for proper sporulation of the isolate.

Colonies are covered with approximately 5 mL of sterile water supplemented with 0.1% Tween-20. Then, the microconidia are carefully rubbed with a sterile cotton swab and transferred with a pipette to a sterile tube. Alternatively, a damp sterile cotton swab could be used to gently touch the culture, and the microconidia transferred to a sterile tube containing 5 mL water supplemented with 0.1% Tween-20. The suspension is vortexed for 15 seconds with a gyratory vortex mixer at approximately 2000 rpm and transferred to a sterile syringe attached to a sterile filter with a pore diameter of 11 μ m, filtered and collected in a sterile tube. This step removes hyphae and yields a suspension composed of microconidia.

The suspension is adjusted with sterile distilled water to 2×10^6 to 5×10^6 microconidia/mL by counting the microconidia in a haemocytometer chamber. Alternatively, a spectrophotometer can be used to adjust the filtered suspension to a concentration equivalent to McFarland 0.5 [21,22]. The suspension is then diluted 1:10 with sterile distilled water to obtain a final working inoculum of 2×10^5 to 5×10^5 CFU/mL [21–24].

Inoculum supplementation with cycloheximide and chloramphenicol

Each inoculum suspension is supplemented with cycloheximide and chloramphenicol in an amount that results in a double-strength final concentration (100 mg/L chloramphenicol and 600 mg/L cycloheximide, respectively). This allows further dilution when the inoculum is added to the test plate, resulting in a final concentration of 50 mg/L chloramphenicol and 300 mg/L cycloheximide in the inoculated plate. In Table 3, examples of a final inoculum of 8 mL are prepared for microdilution plates with four antifungals in a horizontal format, and of 12 mL for a full plate with eight compounds. The volumes can be adjusted to the preferred use in the individual laboratory. The total volume of chloramphenicol and cycloheximide corresponds to 0.8% of the total volume of the inoculum. This will result in a small dilution of the inoculum suspension; but this is below the limit of precision for AFST.

Table 1

The amount of powder or diluent required to prepare a standard solution of antifungal powder, cycloheximide or chloramphenicol may be calculated as follows

$$\text{Weight(g)} = \frac{\text{Volume(L)} \times \text{Concentration(mg/L)}}{\text{Potency(mg/g)}}$$

$$\text{Volume(L)} = \frac{\text{Weight(g)} \times \text{Potency(mg/g)}}{\text{Concentration(mg/L)}}$$

Table 2
Solvents for preparation of stock solutions, characteristics and appropriate test concentration ranges for antifungal agents

Antifungal agent	Solvent	Characteristics	Test range (mg/L)
Amorolfine	DMSO	Hydrophobic	0.008–4
Itraconazole	DMSO	Hydrophobic	0.008–4
Posaconazole	DMSO	Hydrophobic	0.008–4
Terbinafine	DMSO	Hydrophobic	0.004–2
Voriconazole	DMSO	Hydrophobic	0.008–4

DMSO, dimethyl sulfoxide.

Inoculation of microdilution plates

The microdilution plates should be inoculated within 30 min of the preparation of the inoculum suspension to maintain viable microconidia concentration.

The cycloheximide and chloramphenicol supplemented inoculum suspension is vortexed and each well of the microdilution plate is inoculated with 100 µL of the 2×10^5 to 5×10^5 CFU/mL microconidial suspension, without touching the contents of the well. This will give the required final drug concentration and inoculum density (final inoculum 1×10^5 to 2.5×10^5 CFU/mL). The growth control wells (column 11), which contained 100 µL of sterile drug-free medium, are also inoculated with 100 µL of the same inoculum suspension. Column 12 of the microdilution plate is filled with 100 µL of sterile distilled water from the lot used to prepare the inoculum as a sterility control for medium and distilled water (drug-free medium only). Quality control organisms are tested using the same method each time an isolate is tested.

Viability counts should be performed for quality control purposes to ensure that test wells contain between 1×10^5 and 2.5×10^5 CFU/mL, as follows. Ten microlitres of the inoculum suspension should be diluted in 2 mL of sterile distilled water supplemented with 0.1% Tween-20. The suspension is then vortexed with a gyratory vortex mixer at 2000 rpm. Then 100 µL of this suspension is spread over the surface of a suitable agar plate (such as Sabouraud dextrose agar with cycloheximide and chloramphenicol), which is then incubated at 25°C–28°C until colonies can be counted. One hundred to 250 colonies are expected from an acceptable test suspension. A further dilution of 100 µL suspension in 900 µL sterile distilled water supplemented with 0.1% Tween-20, vortexing, and 100 µL plated out would provide an optional/additional count – 10 to 50 colonies would be expected. It is recommended that this is completed for every isolate when the laboratory is setting up this test or conducts the test rarely, when unexplained results are suspected, or periodically (to be locally defined dependent on need).

Incubation of microdilution plates

Microdilution plates are incubated without agitation at 25°C–28°C in ambient air [18]. Most *Trichophyton* isolates should be read at day 5, which was previously found appropriate for

sufficient growth and validated in a multicentre study [18]. A, incubation longer than 7 days is not recommended.

Reading results

During the validation process, spectrophotometric readings (most experience with 490 nm but 405–540 nm applied) using 50% and 90% reduction of the optical density of the growth control when the background was subtracted were compared with visual reading of the plates [18]. However, for itraconazole, trailing growth complicated visual and 90% spectrophotometric inhibition end-point readings. The performance for correct separation of terbinafine wild-type and non-wild-type isolates harbouring target gene mutations was comparable across these end-point methods provided the end-point specific wild-type upper limits were adopted. Hence, the spec-50% end-point was regarded as preferable because it allows an objective end-point determination applicable to the four drugs evaluated. This will avoid subjectivity and lower interlaboratory variation and hopefully facilitate a broader implementation of susceptibility testing of dermatophytes also in laboratories that are less experienced with visual end-point reading.

Interpretation of results

Clinical breakpoints for antifungal agents and dermatophyte species have not yet been established because sufficient MIC and clinical outcome data are not yet available. Interpretation of MICs in the absence of breakpoints is challenging and should be done very carefully taking into account any available data including clinical experience and drug exposure during therapy. However, the MIC may still provide some information regarding susceptibility and, importantly, generation of MICs for dermatophytes is a vital prerequisite for future ECOFF and breakpoint selection. EUCAST established tentative ECOFFs for terbinafine, itraconazole, voriconazole and amorolfine against *T. interdigitale* and *T. rubrum* at the time of establishing this method and they are available at <https://www.eucast.org/astoffungi/>. These may serve to categorize the organism as presumably wild-type or non-wild-type until final ECOFFs and breakpoints are set. Non-wild-type isolates harbour resistance mechanisms and may respond less well to standard therapy.

Quality control

Control procedures are the means by which the quality of results is assured and are described in detail by the CLSI. The routine quality of test results is monitored by the use of control strains.

Control strains

The two *Aspergillus flavus* EUCAST QC strains ATCC 204304 and CLM-CM1813 can be used as quality control of the plates with a 2-day incubation provided the inoculum is prepared without cycloheximide and chloramphenicol supplementation. Of note, the MICs

Table 3

Instructions on calculating the volume needed of chloramphenicol and cycloheximide for addition to the inoculum preparation depending on the final inoculum volume prepared

Inoculum volume	Volume needed of chloramphenicol stock solution (50 000 mg/L)	Volume needed of cycloheximide stock solution (100 000 mg/L)	Total volume added to the inoculum
8 mL	$\frac{100\text{mg/L} \times 8000 \mu\text{L}}{50000\text{mg/L}} = 16 \mu\text{L}$	$\frac{600\text{mg/L} \times 8000 \mu\text{L}}{100000\text{mg/L}} = 48 \mu\text{L}$	64 µL
12 mL	$\frac{100\text{mg/L} \times 12000 \mu\text{L}}{50000\text{mg/L}} = 24 \mu\text{L}$	$\frac{600\text{mg/L} \times 12000 \mu\text{L}}{100000\text{mg/L}} = 72 \mu\text{L}$	96 µL

for the *Aspergillus* strains should be read adopting the visual no growth end-point criterion used for mould testing, as outlined in the E.Def 9.3.2 standard. This will allow a quick quality control of prepared plates, without the need for 5 days of incubation. For quality control of dermatophyte testing, two new quality control strains may be used: *Trichophyton interdigitale* SSI-9396 and *Trichophyton rubrum* SSI-7583. Both strains are wild-type and have tentative MIC targets and ranges established in parallel with the multicentre validation of the method [18]. The recommended MIC target and ranges as well as information on availability of the recommended control strains are available at <https://www.eucast.org/astoffungi/>.

Storage of control strains

Fungal isolates may be stored lyophilized or frozen at -70°C or below [25]. Cultures can be stored short term (<2 weeks) on Sabouraud dextrose agar or potato dextrose agar slopes (*Aspergillus*) at 2°C – 8°C , or Sabouraud dextrose agar supplemented with cycloheximide and chloramphenicol (*Trichophyton* spp.), with new cultures prepared from frozen stocks every 2 weeks.

Routine use of control strains

For routine use of control strains, fresh cultures must be prepared from agar slopes, frozen or lyophilized cultures by inoculation on nutritive agar medium (e.g. Sabouraud dextrose agar or potato dextrose agar for *Aspergillus* spp. or Sabouraud dextrose agar supplemented with cycloheximide and chloramphenicol for *Trichophyton* spp.)

1. At least one control strain must be included per test run and the MICs should be within the control ranges (available at <https://www.eucast.org/astoffungi/>). Two or more strains are needed if the MIC for the quality control strain falls outside the concentration range tested for one or several compounds. If control strain MIC results are out of range, the test should be repeated. If more than one in 20 tests is out of range the source of error must be investigated.
2. Each test must include a well of medium without antifungal drug to demonstrate growth of the test organism and to provide a turbidity control for reading end-points.
3. Subculture inoculum on a suitable agar medium to ensure purity and to provide fresh colonies if re-testing is required.
4. Test each new batch/lot of medium, microdilution plate, and RPMI-1640 2% glucose broth with at least two of the quality control strains (available on the EUCAST website <http://www.EUCAST.org>) to ensure that MICs fall within the expected range.

EUCAST-AFST

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Author contributions

MCA contributed to the conceptualization and wrote the original draft. GK, JM and JG contributed to the conceptualization and to review and editing of the article. All other authors contributed to the review and editing of the article.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.cmi.2020.08.042>.

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