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Clinical laboratory testing and in vitro diagnostic test systems — Reference method for testing the in vitro activity of antimicrobial agents against yeast fungi involved in infectious diseases

Essais de laboratoire clinique et systèmes de diagnostic in vitro — Méthode de référence pour soumettre à essai l'activité in vitro des agents antimicrobiens par rapport aux levures impliquées dans les maladies infectieuses

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Foreword

ISO (the International Organization for Standardization) is a worldwide federation of national standards bodies (ISO member bodies). The work of preparing International Standards is normally carried out through ISO technical committees. Each member body interested in a subject for which a technical committee has been established has the right to be represented on that committee. International organizations, governmental and non-governmental, in liaison with ISO, also take part in the work. ISO collaborates closely with the International Electrotechnical Commission (IEC) on all matters of electrotechnical standardization.

International Standards are drafted in accordance with the rules given in the ISO/IEC Directives, Part 2.

The main task of technical committees is to prepare International Standards. Draft International Standards adopted by the technical committees are circulated to the member bodies for voting. Publication as an International Standard requires approval by at least 75 % of the member bodies casting a vote.

Attention is drawn to the possibility that some of the elements of this document may be the subject of patent rights. ISO shall not be held responsible for identifying any or all such patent rights.

ISO 16256 was prepared by Technical Committee ISO/TC 212, *Clinical laboratory testing and in vitro diagnostic test systems*.

Introduction

In vitro susceptibility tests are performed on microorganisms suspected of causing disease, particularly if the organism is thought to belong to a species that may exhibit acquired resistance to frequently used antimicrobial agents. The tests are also important in resistance surveillance, epidemiological studies of susceptibility and in comparisons of new and existing agents.

Dilution procedures are used to determine the minimum inhibitory concentrations (MICs) of antimicrobial agents and represent the reference method for antifungal susceptibility testing. MIC methods are used in resistance surveillance, comparative testing of new agents for research or registration purposes, to establish the susceptibility of organisms that give equivocal results in routine tests, for tests with organisms where routine tests may be unreliable and when a quantitative result is needed for clinical management. In dilution tests, microorganisms are tested for their ability to produce discernible growth on a series of agar plates (agar dilution) or in broth (broth dilution) containing serial dilutions of the antimicrobial agent.

The lowest concentration of an antimicrobial agent (in mg/l) that, under defined *in vitro* test conditions, reduces visible or optically measurable growth of a microorganism within a defined period of time is known as the MIC. The MIC is a guide for the clinician to the susceptibility of the organism to the antimicrobial agent and aids treatment decisions. Careful control and standardization is required for intra- and inter-laboratory reproducibility, as results may be influenced by the method used. It is generally accepted that broth MIC tests are reproducible to within one doubling dilution of the true end point (i.e. ±1 well or tube in a doubling dilution series).

Broth dilution is a technique in which containers holding identical volumes of broth with antimicrobial agent solutions in incrementally (usually twofold) increasing concentrations are inoculated with a known number of microorganisms.

Broth microdilution denotes the performance of the broth dilution test in microdilution trays.

The reference methods described in this International Standard are intended for the testing of pure cultures of yeast fungi. The broth microdilution methods described in this part of this International Standard are essentially the same as those described by the Clinical and Laboratory Standards Institute (CLSI)[1] and by the European Committee on Antimicrobial Susceptibility Testing (EUCAST)[2]. These methods have been shown to provide MICs of fluconazole that are essentially the same, if not identical up to 2 mg/l[3]. Studies with various other antifungal agents are planned or under way. The laboratory that wishes to use this International Standard for conducting studies of newer antifungal agents, or as a reference method for comparison to MICs generated by a diagnostic device, should select which of the procedure options to use based upon the choice of MIC reading determined by visual inspection (CLSI method) or by use of a spectrophotometer (EUCAST method). In either case, the procedural details for that option are to be followed explicitly.

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WARNING — The use of this International Standard may involve hazardous materials, operations and equipment. This International Standard does not purport to address all of the safety problems associated with its use. It is the responsibility of the user of this International Standard to establish appropriate safety and health practices and determine the applicability of regulatory limitations prior to use.

1 Scope

This International Standard describes a method for testing the susceptibility to antifungal agents of yeasts, including *Candida* spp. and *Cryptococcus neoformans,* that cause infections. The reference method described here has not been used in studies of the yeast forms of dimorphic fungi, such as *B. dermatitidis* and/or *H. capsulatum* variety *capsulatum*. Moreover, testing filamentous fungi (moulds) introduces several additional problems in standardization not addressed by the current procedure. Reference methods for broth dilution antifungal susceptibility testing of filamentous fungi have been developed and are now available as CLSI document M38 and EUCAST document E.DEF 9.1[4][5][6][7][8].

This International Standard describes the broth microdilution reference method which can be implemented by either of two pathways. One pathway involves visual determination of MICs (CLSI method)[1]; the second pathway involves spectrophotometric determination of MICs (EUCAST method)[2]. The MIC reflects the activity of the drug under the described test conditions and can be interpreted for clinical management purposes by taking into account other factors, such as drug pharmacology or antifungal resistance mechanisms. MICs can be categorized as “susceptible” (S), “susceptible dose-dependent” (S-DD), “intermediate” (I), “non-susceptible” (NS) or “resistant” (R). In addition, MIC distributions can be used to define wild type or non-wild type fungal populations. Clinical interpretation of the MIC value is beyond the scope of this International Standard; interpretive category breakpoints specific to the CLSI- and EUCAST-derived methods can be found by consulting the latest interpretive tables provided by the organizations[2][9]. It is advisable to compare routine susceptibility testing methods or diagnostic test devices with this reference method in order to ensure comparable and reliable results for validation or registration purposes.

2 Terms and definitions

For the purposes of this document, the following terms and definitions apply.

2.1
antifungal agent
substance of biological, semi-synthetic or synthetic origin that inhibits the growth of or kills fungi, and is thus of potential use in the treatment of infections

NOTE Disinfectants, antiseptics and preservatives are not included in this definition.

2.2
antifungal agents — properties
2.2.1
potency
active fraction of a test substance, determined in a bioassay against a reference powder of the same substance

NOTE The potency is expressed as mass fraction in milligrams per gram (mg/g), or as activity content in International Units (IU) per gram, or as a volume fraction or mass fraction in percent, or as an amount-of-substance concentration (mass fraction) in mole per litre of ingredients in the test substance.

2.2.2
concentration
amount of an antifungal agent in a defined volume of liquid

NOTE 1 The concentration is expressed as mg/l.

NOTE 2 mg/l = µg/ml but use of the unit µg/ml is not recommended.

2.3
stock solution
initial solution used for further dilutions

2.4
minimum inhibitory concentration
MIC
lowest concentration that, under defined *in vitro* test conditions, reduces growth by an agreed amount within a defined period of time

NOTE The MIC is expressed in mg/l.

2.5
breakpoint
BP
specific MIC values that can be used to assign fungi to the clinical categories “susceptible”, “susceptible dose-dependent,” “intermediate,” “nonsusceptible” and “resistant”

NOTE For current interpretive breakpoints, reference can be made to the latest publications of organizations employing the reference method (e.g. CLSI and EUCAST)[1][2][9].

2.5.1
visual reading pathway
2.5.1.1
susceptible
S
fungal strain inhibited *in vitro* by a concentration of an antifungal agent that is associated with a high likelihood of therapeutic success

NOTE 1 Fungal strains are categorized as susceptible by applying the appropriate breakpoints in a defined phenotypic test system.

NOTE 2 This breakpoint can be altered in certain circumstances (e.g. changes in commonly used drug dosages, emergence of new resistance mechanisms).

2.5.1.2
susceptible dose-dependent
S-DD
fungal strain inhibited *in vitro* by a concentration of an antifungal agent that may be achieved *in vivo* by using higher than normal doses of the agent when such dosage schedules can be safely employed

NOTE 1 Fungal strains are categorized as susceptible dose-dependent by applying the appropriate breakpoints in a defined phenotypic test system.

NOTE 2 This class of susceptibility implies that an infection due to the isolate can be appropriately treated in body sites where the drugs are physiologically concentrated or when a high dosage of drug can be used.

NOTE 3 This breakpoint can be altered in certain circumstances (e.g. changes in commonly used drug dosages, emergence of new resistance mechanisms).

2.5.1.3
intermediate
I
micro-organism having a level of antimicrobial agent activity associated with uncertain therapeutic effect

NOTE This implies that an infection due to the isolate may be appropriately treated in body sites where the drugs are physically concentrated or when a high dosage of drug can be used; it also indicates a buffer zone that should prevent small, uncontrolled, technical factors from causing major discrepancies in interpretations.

2.5.1.4
nonsusceptible
NS
category used for yeast fungi that currently have only a susceptible interpretive category, but not susceptible dose-dependent, intermediate or resistant interpretive categories (i.e. susceptible-only interpretive category)

NOTE This category is often given to new antifungal agents for which no resistant isolates have yet been encountered.

2.5.1.5
resistant
R
fungal strain inhibited *in vitro* by a concentration of an antifungal agent that is associated with a high likelihood of therapeutic failure

NOTE 1 Fungal strains are categorized as resistant by applying the appropriate breakpoints in a defined phenotypic test system.

NOTE 2 This breakpoint can be altered in certain circumstances (e.g. changes in commonly used drug dosages, emergence of new resistance mechanisms).

2.5.2
spectrophotometric reading pathway
2.5.2.1
susceptible
S
micro-organism having a level of antimicrobial activity associated with a high likelihood of therapeutic success

2.5.2.2
intermediate
I
micro-organism having a level of antimicrobial agent activity associated with uncertain therapeutic effect

NOTE This implies that an infection due to the isolate may be appropriately treated in body sites where the drugs are physically concentrated or when a high dosage of drug can be used; it also indicates a buffer zone that should prevent small, uncontrolled, technical factors from causing major discrepancies in interpretations.

2.5.2.3
resistant
R
micro-organism having a level of antimicrobial activity associated with a high likelihood of therapeutic failure

2.6
wild type
absence of acquired resistance mechanisms to the antifungal agent in a given fungal strain

2.7
reference strain
catalogued, well-characterized fungal strain with stable, defined antifungal susceptibility phenotypes and/or genotypes

NOTE Reference strains are kept as stock cultures, from which working cultures are derived. They are obtainable from culture collections and used for quality control.

2.8
susceptibility testing method
2.8.1
broth dilution
technique in which containers are filled with appropriate volumes of an antifungal solution, employing incrementally (usually two-fold) increasing concentrations of the antifungal agent and appropriate volumes of broth with a defined inoculum

NOTE The aim of this method is the determination of the MIC.

2.8.2
microdilution
performance of broth dilution in microdilution trays with a capacity of ≤ 300 µl per well

2.9
broth
fluid medium used for the *in vitro* growth of yeast fungi

2.10
inoculum
number of yeast in a suspension, calculated with respect to the final volume

NOTE The inoculum is expressed as colony-forming units per millilitre (CFU/ml).

2.11
inoculum effect
change in MIC related to change in inoculum

3 Test procedures

3.1 General

The tests are performed in plastic disposable microdilution trays. The method is based on the preparation of double strength antifungal agent working solutions in 100 µl volumes per well with the addition of an inoculum also in a volume of 100 µl.

3.2 Medium

3.2.1 General

RPMI-1640 broth shall be used (see Appendix A for details for preparation of the two versions of RPMI-1640 glucose broth).

3.2.2 Visual reading pathway

The RPMI-1640 medium should contain 0,2 % glucose. The RPMI-1640 broth is prepared and dispensed at single strength with double strength antifungal agent dilutions and the inoculum is delivered in equal volumes of RPMI-1640 broth containing the adjusted yeast inoculum suspension.

3.2.3 Spectrophotometric reading pathway

The RPMI-1640 medium should contain 2 % glucose. The RPMI-1640 broth and antifungal agents are both prepared at double strength with the inoculum subsequently added in an equal volume of sterile distilled water.

3.3 Antifungal agents

3.3.1 General

Antifungal agents shall be obtained directly from the manufacturer or from reliable commercial sources; pharmaceutical preparations for clinical use are not acceptable. The antifungal agents shall be supplied with a lot number, potency, an expiry date and details of recommended storage conditions. Substances shall be stored in tightly closed containers in the dark, at −20 °C, with a desiccant unless otherwise recommended by the manufacturer. Hygroscopic agents should be dispensed into aliquots, one of which is used on each test occasion.

Allow containers to warm to room temperature before opening them in order to avoid condensation and loss of potency.

3.3.2 Preparation of stock solutions

The use of a calibrated analytical balance is required for weighing antifungal agents. Allowance for the potency of the powder shall be made by use of the following formula to obtain the amount of antifungal agent substance or the volume of diluent needed for a standard solution:

$$m = \frac{V \times \rho}{P} \tag{1}$$

$$V = \frac{m \times P}{\rho} \tag{2}$$

where

ρ is the concentration of the stock solution, in mg/l;

m is mass of the antifungal agent (powder), in g;

P is the potency of the antifungal agent (powder), in mg/g;

V is the volume of diluent, in l.

Concentrations of stock solutions should be 1 000 mg/l or greater, although the solubility of some agents is a limiting factor. The actual concentrations of stock solutions depend on the method of preparing working solutions (serial dilutions). Some agents require alternative solvents (see Table 1). Sterilization of solutions is not usually necessary. If required, sterilization should be done by membrane filtration and samples before and after sterilization should be compared by assay to ensure that adsorption has not occurred.

Unless information is available on stability of stock solutions under specified storage conditions, they should be prepared fresh for each test batch.