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Antimicrobial susceptibility testing of *Mycobacterium tuberculosis* complex isolates – the EUCAST broth microdilution reference method for MIC determination

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Abstract (299 words)

Scope: Several methods are used worldwide for antibiotic susceptibility testing (AST) for the *Mycobacterium tuberculosis* complex (MTBC). The variability in the results obtained with these methods hampers to set epidemiological cut-off (ECOFF) values and clinical breakpoints according to EUCAST guidelines. Methods for susceptibility testing and determination of the minimal inhibitory concentrations (MIC) need to be standardized for MTBC isolates for old and new agents. Our objective was to establish a standardized reference method for MIC determination of the MTBC.

Methods: The EUCAST antimycobacterial susceptibility testing subcommittee (AMST) compared protocols of MIC determination with regard to medium, inoculum preparation, antituberculous agent preparation, incubation, reading of the results and interpretation.

Recommendations: The EUCAST reference method of MIC determination for MTBC is the broth microdilution method in Middlebrook 7H9-10%OADC medium. The final inoculum is a 10^5 CFU/ml suspension, from a 10-2 dilution of a 0.5 McFarland suspension prepared after vortexing bacterial colonies with glass beads before suspending them in water. The culture is maintained in a U-shaped 96-well polystyrene microtiter sterile plate with a lid incubated at 36°$\pm$1°C . Reading is done using an inverted mirror as soon as the 1:100 diluted control (i.e. 10^3 CFU/ml) shows visual growth. The MIC, expressed in mg/L, is the lowest concentration which inhibits visual growth. *M. tuberculosis* H37Rv ATCC 27294 is used as the reference strain and its targeted MIC values are within the range 0.03-0.12 for isoniazid, 0.12-0.5 for levofloxacin and 0.25-1 mg/L for amikacin.

Conclusions: The EUCAST reference method for MTBC was endorsed by EUCAST after public consultation and will from now on be used to define EUCAST ECOFFs and clinical breakpoints. This reference method is not primarily intended to be used under routine

conditions and the AST methods need to be calibrated against this reference method to be used with EUCAST breakpoints.

CONTEXT

Since the first antimicrobials were introduced in the treatment of tuberculosis (TB), determinations of minimal inhibitory concentrations (MIC) were performed before human use in order to assess *in vitro* activity against isolates of the *Mycobacterium tuberculosis* complex (MTBC) [1] or in parallel to therapeutics to detect isolates that developed acquired resistance [2]. Although the MIC definition is internationally recognized for all bacteria as the lowest concentration (expressed in µg/ml or mg/L) inhibiting visual growth when compared to a drug-free growth control under the same test conditions, many different methods have been used in the case of antituberculous agents with regards to the inoculum, the medium and the reading endpoints [3]. MIC determination is also a tool used to assess the distribution of clinical isolates according to the antibacterial activity, and such distribution is needed to characterise the epidemiological cut off value (ECOFF). The ECOFF is defined as the highest MIC value measured for phenotypically wild-type isolates; i.e. those without acquired mechanisms of resistance [4]. In the TB field, for the first antituberculous agents such as isoniazid, critical concentrations (CCs) were experimentally determined on microbiological and clinical evidence but mainly on egg-based medium that was used at that time [1]. Several antimicrobial susceptibility testing (AST) methods were developed since the 1960's to separate susceptible (no growth in presence of the antimicrobial agent at CC) and resistant (growth at CC) isolates [3,5]. Many different strategies for AST of MTBC are still used worldwide without reaching a consensus [5], a situation that has complicated methodological standardization and generated several breakpoints per agent [6]. However, clinical breakpoints (CB), as defined for other antimicrobials, are relying also on clinical parameters such as pharmacokinetics, pharmacodynamics and treatment outcome. Consequently, CB should not be dependent on variability with regard to the methods used for AST.

For new antimicrobial agents, EUCAST breakpoints are established during the marketing authorization process conducted by European Medical Agency (EMA) and its Committee for Medical Products for Human Use (CHMP). In 2011, EMA approached EUCAST for determining breakpoints of the new antituberculous, delamanid and bedaquiline, and EUCAST asked the ESCMID study group of mycobacterial infections (ESGMYC) for assistance. The lack of a uniform and standardized reference method for MIC determination of MTBC became obvious as different methods were used during this process. There were inconsistent data to set proper ECOFFs, confusion about which MIC method to use for PK/PD studies, and difficulties with evaluating the level of resistance causing treatment outcome failure [7]. Due to the limited MIC data obtained from several non-standardized methods, provisory breakpoints were set as concentrations of 0.06 mg/L for delamanid and 0.25 mg/L for bedaquiline (www.eucast.org).

In 2016, the EUCAST subcommittee for anti-mycobacterial drug susceptibility testing (AMST) was launched with a primary goal of defining a reference method for MIC determination of the MTBC. Detailed protocols for MIC determination in solid medium (agar dilution in Middlebrook 7H10) and in liquid culture (broth microdilution in Middlebrook 7H9) were developed and evaluated for reproducibility in a multi-centre study for which the results are reported elsewhere.

The aim of this paper is to describe the final protocol for the reference method, addressing some of the questions and comments received during the public consultation. The reference method is intended to be practiced without commercial restrictions.

SCOPE

The EUCAST AMST subcommittee wishes to make the following clarifications regarding the proposed reference MIC method for MTBC. The proposed reference method - broth microdilution in Middlebrook 7H9 media - was set based on the results from a multi-centre study of reproducibility,described elsewhere (Schon et al.) and other considerations such as labour requirements, cost and non-commercially based. The technical protocol was available for consultation during six weeks (from 15th of May to 26th of June, 2019) and then presented to the EUCAST steering committee for a formal decision after considering the necessary amendments resulting from the consultation. The test protocol was published on the EUCAST website on the 19th of July in 2019 as the EUCAST reference method for MIC determination of MTBC. (http://www.eucast.org/documents/consultations/).

The EUCAST reference MIC method is not intended primarily for use in clinical microbiology routine. Within EUCAST, it will be the only method used for defining ECOFFs and clinical breakpoints for MTBC but other AST methods may be used with EUCAST clinical breakpoints as long as they will be calibrated according to the EUCAST AMST recommendations (www.eucast.org). The calibration SOP specifies the requirements and steps for calibrating clinically used methods, commercially-based or not, against the EUCAST reference method.

EUCAST REFERENCE METHOD FOR MIC DETERMINATION OF ANTIMICROBIAL AGENTS FOR *M. TUBERCULOSIS* COMPLEX: STANDARD OF PROCEDURE

The EUCAST reference method is a broth microdilution method similar to EUCAST reference methods for other bacteria and fungi with the exception of the media used, inoculum preparation and time of incubation. Main parameters are described in the Table 1. All *M. tuberculosis* cultures should be maintained according to existing biosafety guidelines [8,9].

Medium

The medium chosen is the commonly used synthetic rich medium, Middlebrook 7H9 (7H9), developed by Dubos and Middlebrook in 1947 [10-12]. Before use, it should be supplemented by a mixture of oleic acid, albumin, dextrose and catalase (OADC), available ready-to-use. Both Middlebrook 7H9 medium and OADC enrichment are available from at least three different manufacturers, as requested by EUCAST guidelines [4]. After the 7H9 broth is prepared from the base according to the manufacturer's instructions and autoclaved, OADC (pre-warmed to room temperature, 18-22°C) is added only if 7H9 has reached 50°C in a pre-warmed water bath. For each 96-well microtiter plate, 10 mL of the ready-made 7H9-OADC broth is needed.

Preparation of the antituberculous agents

All drug solutions should be prepared according with the Good Manufacturing Practice (GMP) and powders must be obtained directly from the drug manufacturer or from reliable commercial sources together with appropriate documentation for quality assurance. Generally, drugs should be dissolved as described in ISO-20776-1 guidelines or if not listed, per recommendation of the manufacturer (see examples in Table 2). Since many antituberculous drugs are not soluble in water, the solvent should be used with caution with regard to its own potential inhibitory effect against MTBC. Consequently, for solvents other than water such as DMSO, there should be growth controls containing the same proportion of solvent (e.g. in case of DMSO) as the drug containing medium, and the concentration of the solvent should be the same for all concentrations.

A stock solution should be prepared as outlined in Table 2, and then aliquoted into 0.2 mL/vials, stored at -20°C or -80ºC as recommended in the CLSI guidelines [12] unless

otherwise specified by the manufacturer. Thawed vials should not be reused. Order and batch number of all agents as well as the date of stock solution preparation should be recorded.

The 4X working solution is prepared with two dilution steps in 7H9-OADC from an aliquot of a stock solution as outlined in Table 2 (example given for isoniazid, levofloxacin and amikacin). The addition of OADC is not necessary for preparing the first working solution but it is necessary for the subsequent dilution step since this will be the final medium solution in which the mycobacteria will grow.

Preparation of microplates

A sterile 96-well U-bottom-shaped polystyrene microtiter plate should be used. Plates made of polypropylene or other plastic material should not be used since mycobacteria and some antituberculous drugs can adhere to the plastic and consequently the concentration may be artificially reduced. When the plates have been prepared, they should be used as soon as possible and within the same day,because the microtiter plates will be incubated for several days at 36°C±1°C and some of the drugs may be inactivated or degraded with regard to the length of the incubation time. For each agent, MIC determination should be done by testing at least 8 concentrations in separated wells to cover the full range of potential MIC values (outlined in Table 2 and Figure 1) with at least one concentration below and one above the MIC target. ECOFFs and breakpoints cannot be established using off-scale MIC values below or above a truncated range of concentrations [13].

As described in the Figure 1, the wells are filled with 0.1 mL 7H9-OADC, except the peripheral wells that are filled with sterile distilled water in order to prevent desiccation during the incubation time. Then, following the plate outline in Figure 1, 0.1 mL of the 4X working solution, which corresponds to the highest concentration of each agent, is added to the left row. It should be ensured that no agent is added to the negative and growth control

(GC) wells. In most cases, a multichannel pipette can be used to make 1:2 dilutions from the highest concentration row to the following row and finally discard the last 0.1 mL of the last row. However, it should be noted that this step is not adequate for the agents for which the solvent should be kept at the same minimum concentration (e.g. 1% DMSO). In this case, the agent working solutions should be diluted separately and each dilution added one by one to the corresponding wells in the microtiter plate.

Preparation of the mycobacterial inoculum

This preparation is a critical step for repeatability and reproducibility since MTBC cultures are spontaneously aggregating, which results in uneven distribution of bacterial cells in the inoculum and dilutions with uncertain final numbers of bacterial cell per mL. Tween80 should not be used as it may affect the drug penetration into the mycobacterial cell wall and potentially reduce the MIC value [14].

For pre-culture, MTBC isolates should be grown on solid media (7H10 or 7H11 Middlebrook agar, Löwenstein-Jensen or other egg-based solid media) and sampled from fresh cultures (within 2 weeks after appearance of visible growth). Approximate 1 mg (4 loops of 1µl or a full 3 mm loop) of morphologically similar colonies, to avoid selecting an atypical variant, will be mixed in a 10-15 mL sterile screw-cap glass tube containing 5-10 sterile glass beads (3 mm) after scratching them along the inside wall of the tube using an applicator stick or plastic loop. It is important to avoid scraping off medium. After careful closing of the cap, it is needed to vortex at least 2 minutes in order to disperse the clumps before adding any liquid. Then, 5 mL of fresh sterile distilled water should be added, the cap is closed tightly and the tube's content homogenized by vigorously vortexing the tube to swirling for at least 2 minutes. It is necessary to wait 30 min for remaining clumps to settle. Adjust the turbidity of the supernatant in a new glass tube to McFarland 0.5 by adding sterile distilled water, and

vortex for 30s. If the suspension density is above McF 0.5, add sterile distilled water until it is reached. If the suspension density is below McF 0.5, it is required to start again from the colonies mixed with o dry glass beads; otherwise colonies will not be sufficiently dissociated. The turbidity of the suspension should be determined using a routine suspension turbidity meter and not by visual estimation.

Preparation of the inoculum dilutions

A 1:100 dilution of the bacterial suspension in 7H9-OADC broth is prepared by two steps of tenfold dilution. The volume of bacterial suspension required for one 96-well microplate is 10 mL. Prepare a 10^{-1} suspension by adding 1 mL of the 0.5 McF bacterial suspension to 9 mL of 7H9-10%OADC and vortex for at least 30s until swirling is obtained. For the 10^{-2} inoculum, add 1 mL of the 10^{-1} suspension to 9 mL of 7H9-OADC. The 10^{-2} suspension will be the growth control (GC100%), whereas a 10^{-4} suspension should be additionally prepared (also in two dilution steps: 1+9 mL for the 10^{-3}, then 1+9 mL for the 10^{-4} suspension). The 10^{-4} suspension will be used as a second growth control (GC1%) for checking the inoculum size and assist in accurately assessing the MIC values.

Controls of the inoculum size

The target is $1x10^5$ CFU/mL from the 10^{-2} dilution of 0.5 McF with an acceptable range from $5x10^4$ to $5x10^5$ CFU/mL for a valid test. The inoculum size is checked by CFU counting on Middlebrook 7H10 agar after plating 10 µl of the 10^{-2} suspension (should grow 500-5000 CFU, i.e. confluent growth), 10 µl of 10^{-3} (50-500 CFU) and 10 µl of 10^{-4} (5-50 CFU) and reading after 21 days incubation at 36°C±1°C. The CFU counting results should be recorded. Plating of control suspensions is better done in duplicate or triplicate for anaccurate estimation of the inoculum size.

Inoculation of the microplate (see Figure 1)

Each antibiotic containing well is complementary filled with 0.1 mL of the 10^{-2} suspension starting by the lowest dilution. The growth controls (GC100% and GC1%) should then be inoculated as outlined in Figure 1.

Incubation

After inoculation, the plate is covered with the plastic lid provided with the sterile microtiter plate and put in a O_2/CO_2-permeable plastic bag or a specific box where a maximum of 3 plates can be stored on top of each other. Incubation conditions for maintaining a temperature of 36°C±1°C and adequate ventilation should be regularly checked. Of notice, the use of plastic sheet sealing was not tested in this protocol and therefore cannot be used without a proper calibration.

Reading and interpretation of results

The plates should be read using an inverted mirror to detect positive and negative growth in the wells. Systematic reading should be scheduled after 7 and 14 days of incubation. If there is still insufficient growth of the GC1% after 14 days, the incubation can last until a maximum of 21 days. As soon as the GC100% and the GC1% are positive (GC1% shows weaker positivity than GC100%), MIC can be determined as the lowest concentration where no visible growth is observed. The 7H9 negative control should show no growth for the test to be valid. Report the MIC value in mg/L.

Quality controls

The fully drug susceptible *M. tuberculosis* H37Rv ATCC 27294 reference strain should be included in each testing round and the same aliquot should not be used beyond five subculture passages.

LIMITATIONS

The intended use of this protocol is MIC determination, primarily for *M. tuberculosis* isolates. Although *M. tuberculosis* is the species most frequently involved in human tuberculosis, the *M. tuberculosis* complex comprises other species, such as *M. bovis*, *M. africanum and M. canettii*, and variants such as *M. bovis* BCG and several lineages of *M. tuberculosis* [15]. These species and lineages may have specific resistance characters, such as intrinsic pyrazinamide resistance of *M. bovis* BCG, or the extra slow growth of *M. africanum*. Consequently, testing strains of these different species and lineages might require amendments to the protocol. Another limitation is that some antituberculous agents may require separate considerations due to their dissolution, stability at 36°C, or other factors. These modifications of the protocol will require constant updates and addenda through the interaction with the EUCAST AMST.

Conflict of interest

All authors declare no conflict of interest regarding this study.

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Authors' contributions: TS, JW, DMC, MV and EC design the study and the reference method. DM, EB, MW, EM participated in the technical challenges of the study. GL, JM, GK, CG, MS participated in writing of the objectives, discussed the results and validated the reference method. All participated in the final discussion. EC wrote a draft of the manuscript and all authors participated in the final version and revisions

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Figure 1. Scheme of the microtiter plate for the EUCAST AMST broth microdilution reference protocol.

AA1, antituberculous agent 1 to be tested ; AA2, antituberculous agent 2; AA3, antituberculous agent 3; GC, growth control; 100% corresponds to the same inoculum as in the drug containing wells; 1% corresponds to the hundredfold diluted inoculum; negative control is 200µl 7H9-OADC ; dH20, sterile distilled water.

	1	2	3	4	5	6	7	8	9	10	11	12
A	200ul	200ul	200ul	200ul	200ul	200ul	200ul	200ul	200ul	200ul	200ul	200ul
	dH20	dH20	dH20	dH20	dH20	dH20	dH20	dH20	dH20	dH20	dH20	dH20
B	negative	GC	AA1 (10-2)	AA1 (10-2)	AA1 (10-2)	AA1 (10-2)	AA1 (10-2)	AA1 (10-2)	AA1 (10-2)	AA1 (10-2)	GC	200ul
	control	100%	C8	C7	C6	C5	C4	C3	C2	C1	1%	dH20
C	negative	GC	AA1 (10-2)	AA1 (10-2)	AA1 (10-2)	AA1 (10-2)	AA1 (10-2)	AA1 (10-2)	AA1 (10-2)	AA1 (10-2)	GC	200ul
	control	100%	C8	C7	C6	C5	C4	C3	C2	C1	1%	dH20
D	negative	GC	AA2 (10-2)	AA2 (10-2)	AA2 (10-2)	AA2 (10-2)	AA2 (10-2)	AA2 (10-2)	AA2 (10-2)	AA2 (10-2)	GC	200ul
	control	100%	C8	C7	C6	C5	C4	C3	C2	C1	1%	dH20
E	negative	GC	AA2 (10-2)	AA2 (10-2)	AA2 (10-2)	AA2 (10-2)	AA2 (10-2)	AA2 (10-2)	AA2 (10-2)	AA2 (10-2)	GC	200ul
	control	1%	C8	C7	C6	C5	C4	C3	C2	C1	100%	dH20
F	negative	GC	AA3 (10-2)	AA3 (10-2)	AA3 (10-2)	AA3(10-2)	AA3 (10-2)	AA3 (10-2)	AA3 (10-2)	AA3 (10-2)	GC	200ul
	control	1%	C8	C7	C6	C5	C4	C3	C2	C1	100%	dH20
G	negative	GC	AA3 (10-2)	AA3 (10-2)	AA3 (10-2)	AA3(10-2)	AA3 (10-2)	AA3 (10-2)	AA3 (10-2)	AA3 (10-2)	GC	200ul
	control	1%	C8	C7	C6	C5	C4	C3	C2	C1	100%	dH20
H	200ul	200ul	200ul	200ul	200ul	200ul	200ul	200ul	200ul	200ul	200ul	200ul
	dH20	dH20	dH20	dH20	dH20	dH20	dH20	dH20	dH20	dH20	dH20	dH20

Table 1. Summary of the EUCAST ASMT reference method standard operational procedures (SOP) for MIC determination of antituberculous agents

Parameters	EUCAST AMST SOP	Comment
Medium	Middlebrook 7H9 + 10% OADC	
Plate	U-shaped polystyrene microtiter sterile plate	Covered by a lid*
Pre-culture	Solid media: egg based (LJ, Coletsos…) or synthetic (7H10, 7H11)	Harvest colonies no later than 2 weeks after visible growth
Inoculum	10^{-2} dilution of a McF 0.5 suspension (two steps dilution)	10^5 CFU/mL final concentration
Incubation	36 $\pm$ 1°C up to 21 days in ambient air	Plates are placed in a specific box with sterile water
Reading	Visual reading using an inverted mirror	In safety cabinets and following BSL3 safety measures
Time of reading	As soon as the 1:100 diluted control (i.e. 10^3 CFU/ml) shows visual growth	Between 7 to 21 days
Report of MIC	Lowest concentration where no visual growth	Value in mg/L
Quality control	*M. tuberculosis* H37Rv ATCC 27294	Isoniazid MIC: 0.03-0.12 Levofloxacin MIC: 0.12-0.5 Amikacin MIC: 0.25-1

* The sealing with a plastic sheet was not tested and should be calibrated if prefered

Table 2. Examples of preparation of anti-tuberculous agents that were evaluated using the reference protocol by EUCAST-AMST against *M. tuberculosis* H37Rv ATCC 27294

Antimicrobial agent	Sigma ref for drug powder No	Solvent	Stock conc[b] (mg/L)	Dilution 1 (7H9)[c]	Dilution 2 (7H9/OADC)	Working conc. in 7H9/OADC (mg/L)[d]	Range of final concentrations (mg/L)
Isoniazid	I3377	dH_20	10 240	1:64	1:40	4	1-0.008
Amikacin	A1774	dH_20	10 240	1:64	1:5	32	8-0.06
Levofloxacin	28266	NaOH[a]	10 240	1:64	1:10	16	4-0.03

[a]Add powder to 50% dH_20 of the total volume and then 1 mol/L NaOH dropwise to dissolve. Then add dH_20 to the final volume.

[b]Calculate the amount of drug to dissolve in 10 mL according to potency: m = V*p/P. m=mass of the antimicrobial agent (powder) in g; p=concentration of the stock solution in mg/L; P=potency of the antimicrobial agent (powder) in mg/g (ie 67% potency means 670mg/g or and 100% potency 1000 mg/g); V=volume of solvent in Liter. In case the potency is not specified by the manufacturer, it may be calculated by (assay purity) x (active fraction) x (1 – water content) , i.e. for a 99.8% purity, 12,1% water content and 100% active fraction the potency is (998) x (1.0) x (1– 0.121) = 877 µg/mg or 87.7%

[c]The addition of OADC is not necessary in this step as it is for further dilution only.

[d] 1mL is needed for 10 plates