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## Research note

### Multicentre testing of the EUCAST broth microdilution reference method for MIC determination on *Mycobacterium tuberculosis*

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## ABSTRACT

**Objectives:** The first objective of the European Committee on Antimicrobial Susceptibility Testing (EUCAST) subcommittee for antimycobacterial susceptibility testing (AMST), launched in 2016, was to set a reference method for determining the MICs of antituberculous agents, since many protocols are used worldwide and a consensus one is needed for the determination of microbiological breakpoints.

**Methods:** During 2017 and 2018, MIC determination protocols were evaluated prospectively in a multicentre study within the four AMST laboratories. MIC results were obtained for isoniazid, levofloxacin and amikacin on the reference strain *Mycobacterium tuberculosis* H37Rv ATCC 27294. Broth microdilution (BMD) in Middlebrook 7H9 and solid medium dilution (SMD) in Middlebrook 7H10 were performed using two inoculum concentrations. MICs were interpreted with regard to visual and 99% inhibition after 7, 14 or 21 days of incubation for BMD and 21 days for SMD.

**Results:** Following the EUCAST reference protocol, intra- and inter-assay agreements were within  $\pm 1$  MIC dilution for >95% of the observations for the three drugs in both methods. MIC values, presented as MIC mode (range) for BMD and SMD respectively, were: 0.03 (0.015–0.06) mg/L and 0.12 (0.06–0.25) mg/L for isoniazid, 0.25 mg/L (0.25–0.5) and 0.5 mg/L (0.12–0.5) for levofloxacin, and 0.5 mg/L (0.5–1.0) and 0.5 mg/L (0.5–1.0) for amikacin.

**Conclusions:** Both SMD and BMD were reproducible and eligible as a reference method for MIC determination of the *Mycobacterium tuberculosis* complex (MTBC). BMD was finally selected as the EUCAST reference method. From now on it will be used to set epidemiological cut-off values and clinical breakpoints of new and old antituberculous agents. **Thomas Schön, Clin Microbiol Infect 2021;27:288.e1–288.e4**

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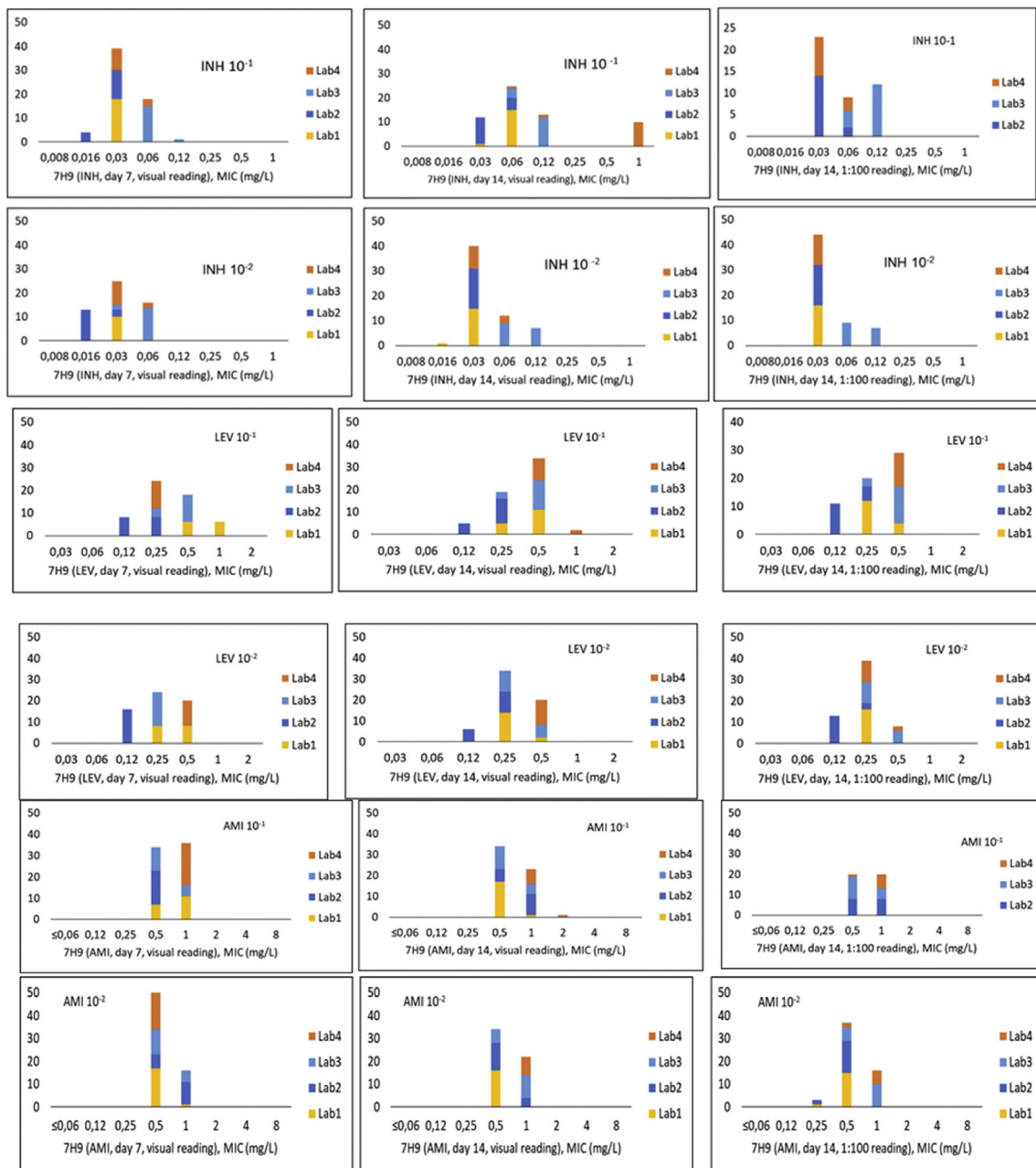
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## Introduction

Several methods have been described for the determination of MIC of antituberculous agents against the *Mycobacterium tuberculosis* complex (MTBC) [1]. In contrast to most other bacteria, bacteria of the MTBC pose challenging technical issues since (a) they are slow-growing (several days to several weeks) in enriched media which differ from the traditionally used Mueller–Hinton, (b) they produce clumps when growing in liquid and solid media, and (c) the antimicrobials are often specific, which precludes the use of control bacteria from other species. A stable reference method for MIC testing of the MTBC is required to define epidemiological cut-off values and clinical breakpoints according to the

European Committee on Antimicrobial Susceptibility Testing (EUCAST) strategies, but also to facilitate studies on the relationship between resistance mutations predicted from sequencing data in relation to the MIC level of resistance. In 2016, the EUCAST subcommittee for antimycobacterial drug susceptibility testing (AMST) was launched with a primary goal of defining a reference method for MIC determination on the MTBC. This reference method was made publicly available on the EUCAST website in July 2019 ([https://www.eucast.org/mycobacteria/methods\\_in\\_mycobacteria/](https://www.eucast.org/mycobacteria/methods_in_mycobacteria/)) and published [2] along with a comment [3]. Herein, we report the results obtained by the AMST laboratories when testing various protocols with the objective of agreeing on one protocol that produces MIC results with good reproducibility.



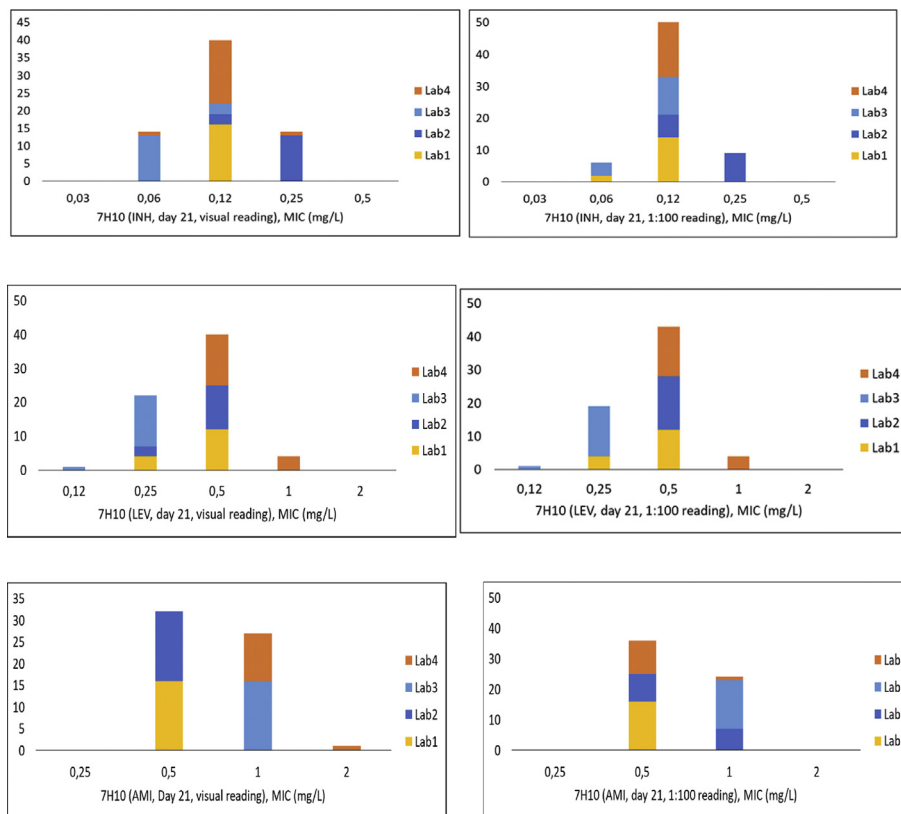
**Fig. 1.** Results of inter-laboratory testing with the broth (7H9) microdilution method for isoniazid (INH), levofloxacin (LEV), and amikacin (AMI) using two different inoculum sizes, 10<sup>-1</sup> and 10<sup>-2</sup> of a McF 0.5 suspension of *Mycobacterium tuberculosis* H37Rv ATCC 27294.

## Methods

MICs of isoniazid, levofloxacin and amikacin were determined for *M. tuberculosis* H37Rv ATCC 27294 maintained in each laboratory. Middlebrook 7H9 broth microdilution (BMD) was tested with a high and low inoculum (1:10 and 1:100 dilutions of a controlled McFarland (McF) 0.5 suspension) in comparison with the proportion method onto Middlebrook 7H10 solid medium (SMD). BMD was performed according to the EUCAST reference method [2]. The SMD protocol was derived from Clinical and Laboratory Standards Institute (CLSI) [4] with modifications detailed below: the inoculum was prepared as described in the EUCAST reference method but adjusted to McF1.0, and 100  $\mu$ L of  $10^{-2}$  and  $10^{-4}$  dilutions were plated in duplicate onto plates filled in with 7H10-10%OADC. Reading was performed after 7, 14 and 21 days' incubation for BMD using an inverted mirror and 21 days for SMD. The MIC was the lowest concentration without visual growth using two strategies. SMD was read using both 1:1 and 1:100 diluted controls according to the CLSI. BMD was read for visual growth when the 1:100 dilution growth control was positive, according to the EUCAST reference method [2]. Intra- and inter-laboratory agreement for BMD and SMD were calculated for MIC values  $\pm 1$  MIC dilution of the mode, measured in quadruplicates and repeated in at least four separate experiments in each laboratory. The antibiotic stability in 7H9 media was measured at D0, D1, D3 and D14 for isoniazid at 0.5 mg/L (Clinical Pharmacology, Huddinge Hospital, Sweden), for levofloxacin at 1 mg/L by LC/MS-MS (Clinical Pharmacology, Groningen, The Netherlands) and for amikacin at 2 mg/L by immunochromatography (Lund University Hospital, Sweden). All laboratories are accredited for the respective analyses.

## Results

MIC distributions obtained from each laboratory using the BMD protocol ( $n = 1623$ ) are presented in Fig. 1 and those for the SMD protocol ( $n = 390$ ) in Fig. 2. For SMD, there was a 99% (range: 98–100%) intra- and 99.5% (98.5–100%) inter-laboratory agreement for MICs. For BMD, the intra-laboratory agreement was 100% for both inocula in almost all conditions at days 7 and 14, and the inter-laboratory agreement at those time points was 100% and 96% (88–100) respectively, using the  $10^{-2}$  dilution inoculum, whereas it was 96% (89–100%) and 85% (71–98%), respectively for the  $10^{-1}$  dilution. No significant differences in the MIC distributions were observed in BMD while comparing visual reading and the use of a 1:100 diluted control for the reference except for levofloxacin after 14 days' incubation. Mean levofloxacin MIC values were different (0.32 for visual reading and 0.26 for the 1:100 reading,  $p = 0.015$  using the Mann–Whitney U test) and a slight right shift in the MICs was observed for visual reading (Fig. 1). Median MIC and MIC range were, however, the same at 0.25 and 0.12–0.5 mg/L, respectively. Median MICs of all drugs were shifted to the twofold higher value in BMD when incubation exceeded 14 days: 0.06 (0.03–1.0) at day 21 versus 0.03 (0.015–0.25) mg/L at day 14 for isoniazid and 0.5 (0.12–1.0) versus 0.25 (0.06–0.5) mg/L for levofloxacin, respectively. After 21 days of incubation, the Gaussian shape of the distributions was distorted (Supplementary Fig S1). In accordance with the EUCAST reference method [2], the first reading was done at day 7, but in two out of four laboratories the 1:100 control did not show sufficient growth at this time, whereas all laboratories could read the MIC results at day 14. Median MICs were significantly higher for isoniazid (0.12 [0.06–0.25] versus 0.03 [0.015–0.12] mg/



**Fig. 2.** Results of inter-laboratory testing with the solid medium (7H10) dilution method for isoniazid (INH), levofloxacin (LEV), and amikacin (AMI) using two different inoculum sizes,  $10^{-2}$  and  $10^{-4}$  of McF 1.0 suspension of *Mycobacterium tuberculosis* H37Rv ATCC 27294.

L) and levofloxacin (0.5 [0.25–1.0] versus 0.25 [0.12–0.5] mg/L) but not amikacin (0.5 [0.5–2.0] versus 0.5 [0.5–1.0] mg/L) in SMD compared to BMD.

Inoculum concentration for BMD, measured as the mean of 33 determinations, was  $1.7 \times 10^5$  CFU/mL (range  $1.0 \times 10^4$  CFU/mL to  $5.8 \times 10^5$  CFU/mL). For SMD, the mean inoculum was  $2.8 \times 10^6$  CFU/mL (range  $1.0 \times 10^5$  CFU/mL to  $1.1 \times 10^7$  CFU/mL,  $n = 72$ ). The results of antibiotic concentration measurements were 0.53, 0.49, 0.46 and 0.45 mg/L, respectively, at days 0, 1, 3 and 14 for isoniazid and for levofloxacin (1, 1, 1 and 1.1 mg/L) and finally amikacin (2.6, 2.3, 2.5 and 2.7 mg/L).

## Discussion

We evaluated optimized protocols for MIC determination on the *M. tuberculosis* complex following EUCAST methodology and principles [5]. The objective was to provide a reproducible MIC method for setting epidemiological cut-off values. Thus, we prioritized repeated MIC testing of the reference strain *M. tuberculosis* H37Rv ATCC 27294 only for selecting the most suitable reference method from a reproducibility perspective [4]. High intra- and inter-laboratory agreements were achieved after experimental testing of different inoculum sizes, which confirmed that one of the limiting factors in achieving reproducibility is the inoculum preparation, which requires a tight control of the final concentration of the bacterial cells tested [6,7].

Another critical factor is the preparation of the stock solution of the antituberculous agent and the dilution steps towards the final concentrations. For this first AMST testing, we chose only three of the main antituberculous agents, and especially the ones that are water-soluble to avoid the potential effect of other solvents. Despite this, we observed discrepancies in the MIC values until we carefully adjusted the drug potency for amikacin, and the NaOH molarity of the water solution for levofloxacin. Since the incubation is long, to enable the slow growth of MTBC isolates, antibiotic instability may affect the final MIC value, and that was shown when reading after 21 days' incubation. This is well known in egg-based media that need to be coagulated at temperatures  $>60^\circ\text{C}$  before inoculation, and for which antituberculous agent concentrations are often 10- to 20-fold higher than in other media [8]. Although we showed measurable levels of isoniazid, amikacin and levofloxacin throughout incubation in this study, this will have to be confirmed in future studies including other antituberculous agents.

After strictly following the BMD reference protocol, we obtained MIC values that did not differ more than  $\pm 1$  dilution within and between laboratories, and showed less variability than in previous multicentre studies following other protocols [9]. Overall, SMD and BMD produced similar results, but the hands-on-time for preparation, inoculation and reading was much longer for SMD than for BMD [2]; the considerations for selecting the reference protocol are described elsewhere [3].

## Author contributions

TS, JW, DMC, MV and EC designed the study. 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 them. All participated in the final discussion. EC wrote a draft of the manuscript and all authors participated in the final version and revisions.

## Transparency declaration

All authors declare no conflict of interest regarding this study. This study was supported by a grant from ESCMID to the study group ESGMYC (ESCMID study group on mycobacterial infections) 2017–2019.

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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.2020.10.019>.

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