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COMMENTARY

On the Consequences of Poorly Defined Breakpoints for Rifampin Susceptibility Testing of *Mycobacterium tuberculosis* Complex

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ABSTRACT In a recent report of a systematic review of critical concentrations (CCs), the World Health Organization (WHO) lowered the rifampin (RIF) CC for antimicrobial susceptibility testing (AST) of the *Mycobacterium tuberculosis* complex using Middlebrook 7H10 medium and the Bactec Mycobacterial Growth Indicator Tube (MGIT) 960 system from 1 to 0.5 μg/ml. The previous RIF CC for 7H10 had been in use for over half a century. Because it had served as the *de facto* reference standard, it contributed to the endorsement of inappropriately high CCs for other AST methods, including the U.S. Food and Drug Administration (FDA)-approved MGIT system. Moreover, this resulted in confusion about the interpretation of seven borderline resistance mutations in *rpoB* (i.e., L430P, D435Y, H445L, H445N, H445S, L452P, and I491F). In this issue of the *Journal of Clinical Microbiology*, Shea et al. (J Clin Microbiol 59:e01885-20, 2021, https://doi.org/10.1128/JCM.01885-20) provide evidence that the CC endorsed by the Clinical and Laboratory Standards Institute for the Sensititre MYCOTB system, which is not FDA approved but is CE-IVD marked in the European Union, is likely also too high. These findings underscore the importance of calibrating AST methods against a rigorously defined reference standard, as recently proposed by the European Committee on Antimicrobial Susceptibility Testing, as well as the value of routine next-generation sequencing for investigating discordant AST results.

Rifampin (RIF) first became available in the late 1960s and was initially used for retreatment of drug-resistant tuberculosis (TB) (1). It is now recognized as the most important anti-TB agent, as it has reduced the treatment duration of drug-susceptible TB from 18 to 9 months and, ultimately, to 6 months following the inclusion of pyrazinamide in the 1970s (2). However, its value has been eroded through the widespread emergence and transmission of RIF-resistant strains. Indeed, approximately half a million people developed RIF-resistant TB in 2019, of whom 78% had multidrug-resistant TB (MDR-TB) (3).

In 1993, Telenti and coauthors demonstrated that mutations in the RNA polymerase subunit β, encoded by *rpoB*, confer RIF resistance in the *Mycobacterium tuberculosis* complex (MTBC) (4). This landmark study, and subsequent work to identify and characterize the various resistance mutations in *rpoB*, paved the way for the development of genotypic assays that have shortened the time required for antimicrobial susceptibility testing (AST) from weeks to days or hours (5). Nevertheless, only 61% of bacteriologically confirmed pulmonary TB cases were tested for RIF resistance in 2019 compared to the target of 100% set by the World Health Organization (WHO) in the END TB Strategy (3). More fundamentally, the molecular understanding of resistance provided crucial evidence that RIF resistance had not

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been defined rigorously on the phenotypic level for more than half a century, complicating routine diagnostics and patient treatment (6, 7).

PHENOTYPIC AST FOR RIF

The epidemiological cutoff value (ECV) as a conservative clinical breakpoint. Phenotypic AST relies on clinical breakpoints that distinguish samples that are susceptible at the standard dosing regimen of a drug because they have a high likelihood of therapeutic success compared to those that do not and, consequently, are resistant (8). For some agents, the European Committee on Antimicrobial Susceptibility Testing (EUCAST) has introduced the third category, I, as "susceptible, increased exposure" (i.e., a microorganism is categorized as I when there is a high likelihood of therapeutic success, because exposure to the agent is increased by adjusting the dosing regimen or by its concentration at the site of infection, which differs from "intermediate" as defined by the Clinical and Laboratory Standards Institute [CLSI] [9, 10]).

When an antibiotic that does not share any resistance mechanism with a previously used agent is first evaluated, the MICs of that drug usually form a normal distribution that captures both the limited preexisting biological variability and the technical variability of MIC testing (i.e., no major differences in the intrinsic resistance typically exist, although exceptions are possible, as is the case for pyrazinamide and thioacetazone [8, 11, 12]). The upper end of this distribution of phenotypically wild-type (pWT) strains corresponds to the ECV, which is referred to as the critical concentration (CC) in the TB field (13, 14). Although pharmacokinetic (PK) and pharmacodynamic (PD) data have not been satisfactorily evaluated to date by either CLSI or WHO, there is convincing clinical evidence for the efficacy of RIF against pWT strains, which means that these are susceptible and the ECV can serve as a clinical breakpoint, as defined by EUCAST (2). Conversely, any strains with elevated MICs (i.e., that are phenotypically non-wild type [pNWT]) are assumed to be resistant, in accordance with the precautionary principle until sufficient evidence to the contrary becomes available.

Inappropriately high critical concentrations. Despite the importance of the ECV for phenotypic AST, no ECV for any drug has ever been set for MTBC that would meet modern microbiological principles because of the dearth of high-quality MIC data (15, 16). For example, RIF MICs are typically truncated, which precludes a comprehensive assessment of the pWT MIC distribution. In addition, systematic differences exist between laboratories, as some methods are not sufficiently standardized (17). More fundamentally, until recently regulators had not collected and systematically analyzed existing data and, instead, largely relied on expert opinion and historical precedent to set CCs (15). For example, it was not until 2020, more than a decade after endorsing 1 μg/ml as the RIF CC for the Bactec Mycobacterial Growth Indicator Tube 960 (MGIT) system by Becton Dickinson, as well the noncommercial Middlebrook media 7H10 and 7H11, that the WHO reviewed these concentrations systematically (7, 18).

The limited quality and quantity of RIF MIC data notwithstanding, this review revealed that the currently CLSI-endorsed and previously WHO-endorsed RIF CCs for 7H10 and MGIT are actually one dilution higher than the tentative ECV, which also calls into question the CC for 7H11 given the similarity of this medium to 7H10 (7). Taking PK/PD and clinical outcome data into consideration, WHO adopted a cautious approach by lowering the RIF CCs of 1 μg/ml for 7H10 and MGIT to the tentative ECVs of 0.5 μg/ml for both media (7). The RIF CC for Löwenstein-Jensen (LJ) remained unchanged at 40 μg/ml, as insufficient MIC data were identified to assess the shape of the pWT MIC distribution, even though the CC for this medium had been adopted by WHO in 1969 and reaffirmed by CLSI in 2018 (7, 19, 20).

The RIF CC for 7H10 appears to have been originally set at 1 μg/ml by the U.S. Centers for Disease Control and Prevention (CDC) in 1969 based on the MICs of just 20 pWT strains that varied between 0.05 and 0.2 μg/ml (21). Notably, no rationale was provided for choosing 1 μg/ml instead of 0.2 μg/ml. Setting the breakpoint at the higher concentration essentially deemed some pNWT strains with elevated MICs treatable with RIF. In 1974, this CC was included in the second edition of the American

Society for Microbiology *Manual of Clinical Microbiology* and featured in all subsequent editions (22). In the third edition of the same manual from 1980, 1 μg/ml was adopted as the CC for 7H11, although it is not clear what evidence led to this decision (23). Similarly, it is not apparent from the first CLSI guidelines that adopted both CCs in 2003 whether the CLSI committee reviewed any data underlying the CDC decision to set the 7H10 CC above the tentative ECV (24). Either way, the RIF CCs for 7H10 and 7H11 were reaffirmed in all subsequent CLSI guidelines, and the 7H10 CC was used as part of the U.S. Food and Drug Administration (FDA) approval of MGIT and the VersaTREK Myco susceptibility kit by Thermo Fisher.

In this issue of the *Journal of Clinical Microbiology*, Shea et al. add to this picture by providing evidence that the CLSI-endorsed RIF CC for the Sensititre MYCOTB broth microdilution plate by Thermo Fisher, which is not FDA approved but is CE-IVD marked in the European Union, is likely set too high (6, 17). Specifically, their findings are in line with an earlier study by Torrea et al. and indicate that the CC needs to be lowered to the tentative ECV of 0.5 μg/ml, following a more detailed review of additional data sets (25).

Borderline resistance mutations and areas of technical uncertainty (ATUs). It is important to acknowledge that setting a CC one dilution above the ECV has an advantage. Specifically, it minimizes random false resistance results linked to the fact that the ECV is set to encompass 99% of pWT strains (i.e., in a setting with only susceptible strains, approximately 1% would be misclassified as resistant, resulting in a poor positive predictive value of phenotypic AST). Such major testing errors are a particular concern for RIF, as they often trigger treatment with MDR-TB regimens that are considerably more toxic and less effective than drug-susceptible TB treatment regimens. The disadvantage of testing at a higher breakpoint is that it increases the risk of missing resistant strains that have MICs close to the ECV. This possibility went largely unnoticed for three main reasons. First, phenotypic AST for MTBC is typically carried out only at the CC, yielding categorical rather than quantitative results (i.e., a strain is simply classified a susceptible or resistant, as no MIC testing is carried out). Second, repeat testing or testing with multiple phenotypic methods is rarely done. Third, such borderline resistant strains are relatively infrequent in countries with comprehensive laboratory capacity to investigate this phenomenon, although the prevalence of these strains has been underestimated because of the inappropriately high CCs, as outlined above.

It was not until the discovery of the RIF resistance-determining region (RRDR), which spans codons 426 to 452 of *rpoB*, that a set of strains with mutations were noticed that had poor AST reproducibility at the CC, particularly in MGIT (4, 7, 26). These mutations have caused considerable confusion in the literature and have been referred to as "borderline resistant," "discordant," "disputed," "occult," or "(subbreakpoint) low-level resistance" mutations. Specifically, there was no consensus on whether some of these might be genuinely neutral (i.e., do not confer elevated MICs, as is the case for at least one nonsynonymous mutation in the quinolone resistance-determining region of *gyrA* [27]), and, if they do increase the MIC, why these have poor reproducibility and whether they are clinically relevant. In fact, there was no agreement even on which mutations fall into this category.

As part of the aforementioned systematic review, WHO investigated all of these questions (7). WHO found that six RRDR mutations (i.e., L430P, D435Y, H445L, H445N, H445S, and L452P) and the non-RRDR I491F change correlated with MIC distributions that overlap the upper part of the MIC distributions of pWT strains. Therefore, once the CC is lowered to the tentative ECVs endorsed by WHO, the reproducibility of categorical AST will improve but still remain poor compared with mutations that confer larger MIC increases, such as the high-level resistance mutation *rpoB* S450L (Fig. 1). This applies to the MYCOTB plate and all WHO-endorsed media, including LJ (i.e., it is a misconception that LJ is a reliable confirmatory test for borderline resistance mutations) (6, 25). It should be noted, however, that even when using the new WHO CCs, the rate of misclassification of these borderline resistance mutations as susceptible will likely remain higher for MGIT than for LJ and 7H10, which could be due to one of two

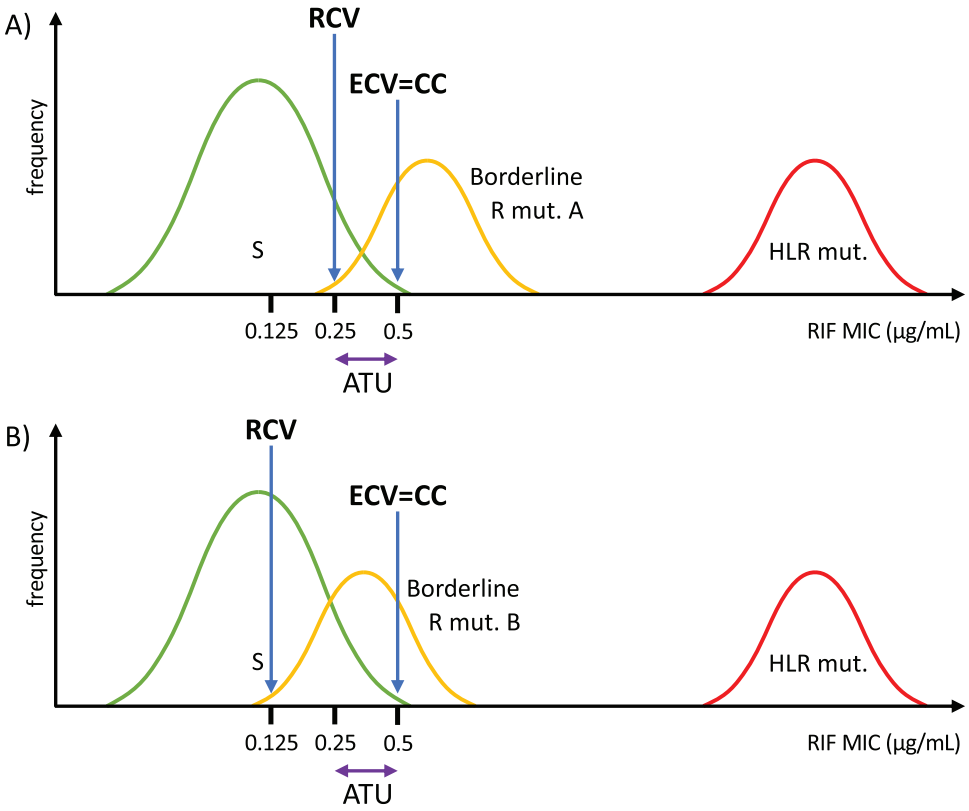

FIG 1 Potential role for an area of technical uncertainty (ATU) for RIF AST. These diagrams show the idealized MIC distributions for susceptible (S) strains compared with strains with two different borderline resistance (R) mutations and a high-level resistance (HLR) mutation. Because HLR mutations confer large MIC increases, these mutations have a good reproducibility for categorical AST at the critical concentration (CC), which corresponds to the epidemiological cutoff value (ECV). In contrast, borderline resistance mutations have a good essential agreement but poor categorical agreement, as their MIC distribution is divided by the CC (i.e., even when the same isolate is tested repeatedly in the same laboratory under carefully controlled conditions, it will variably test susceptible and resistant due to the inherent technical variability of phenotypic AST, where the ratio of susceptible to resistant results depends on the degree of overlap with the susceptible MIC distribution [9, 10, 14, 29]). In plot A, an ATU could be set at 0.5 μg/ml to signal that MICs at this concentration cannot be unequivocally classified as susceptible or resistant (i.e., that the interpretation is uncertain based on a single RIF MIC result [30]). Although the prevalence of the corresponding borderline resistance mutation A in a particular setting can give an indication of which of these possibilities is more likely, other experimental results are needed to resolve this situation conclusively. For example, if this borderline mutation is detected by genotypic AST, the isolate should be reported as resistant, as recommended by WHO (7). In plot B, the MIC distribution of the borderline resistance mutation B overlaps to a greater degree with the susceptible population. This cannot be compensated for by extending the ATU to the resistant cutoff value (RCV) for this mutation (i.e., the lower end of this mutation, which would be 0.125 μg/ml), given that this would classify too many susceptible isolates as uncertain, particularly if the isolate is otherwise pan-susceptible (29, 31, 32). Nevertheless, resistance can be ruled in reliably by the detection of the corresponding borderline mutation B.

reasons. First, the new MGIT CC may still be one dilution above the true ECV, which should be clarified as a matter of urgency (7). Second, there might genuinely be a greater overlap between the MIC distributions of susceptible strains and borderline resistant strains with MGIT because of the shorter incubation period of this method using standard conditions (i.e., if the incubation period for MGIT is extended, the MICs of borderline resistant strains increase more than those of susceptible strains, thereby reducing the overlap of the MIC distributions [25]).

WHO also assessed PK/PD data for RIF and the limited clinical outcome data available for these seven *rpoB* mutations. In light of that limited direct and indirect evidence and to err on the side of caution, WHO recommended that these mutations be regarded as clinically relevant for the current 10 mg/kg of body weight/day dose of RIF (7). In fact, some of the perceived impacts of isoniazid monoresistant TB are likely due to undiagnosed borderline RIF

resistance (28). As a result, WHO decided to refer to these seven mutations as "borderline resistance" mutations to emphasize that these are clinically relevant and display a poor reproducibility for categorical phenotypic AST (i.e., as opposed to, for example, using the term "low-level resistance," which does not capture the reproducibility aspect and might be interpreted as a signal that sufficient evidence exists that an increased dose of RIF overcomes this level of resistance). WHO acknowledged that this decision would have to be reassessed in light of new evidence, especially should a higher dose of RIF be endorsed (7). Indeed, this question is particularly pressing for patients with otherwise pan-susceptible TB, for whom a dose adjustment for RIF, ideally accompanied by therapeutic drug monitoring for all first-line drugs, might be a better option than to provide a full MDR-TB regimen.

WHO declined to set ATUs, as recently introduced by EUCAST, to reduce very major testing errors for several bacterial pathogens due to overlapping MIC distributions (7, 9, 29, 30). The findings by Shea et al. underline that this could be implemented easily for the MYCOTB plate but would not fully eliminate very major errors for borderline resistance mutations that have resistant cutoff values that are more than one dilution below the ECV (Fig. 1) (31, 32). Indeed, CLSI has already set an "inconclusive" category for ethambutol on the MYCOTB plate that appears to serve a function similar, if not identical, to that of the ATU, even though CLSI does not recognize the concept of an ATU for other bacteria (9, 33).

GENOTYPIC AST FOR RIF

WHO expert rule. Countries that rely only on phenotypic AST, particularly if only the CC is tested, will miss a proportion of borderline RIF-resistant strains. Therefore, genotypic AST is not merely an approach to accelerate AST but represents the reference standard for the seven borderline resistance mutations. Indeed, WHO does not recommend confirmatory testing with phenotypic AST for strains with these mutations (7). When confirmatory phenotypic AST is nonetheless conducted (e.g., because clinicians decide which testing is carried out or it is mandated by insurers), WHO recommends that the detection of a borderline resistance mutation should overrule a susceptible phenotypic AST result, provided that the pretest probability has been considered and obvious laboratory or clerical errors have been excluded (i.e., if there is a concern regarding the validity of the genotypic result, a reliable genotypic AST method is the appropriate confirmatory approach).

Moreover, WHO reaffirmed an expert rule first introduced in 2018 that any mutation in RRDR, with the exception of synonymous mutations, should be assumed to confirm RIF resistance and overrule a susceptible phenotypic AST result, as novel RRDR mutations might behave like the seven known borderline resistance mutations (7). In other words, a composite reference standard was endorsed, whereby a strain is considered resistant if it tests phenotypically resistant, has a known resistance mutation (i.e., one of the seven borderline resistance mutations or an *rpoB* mutation satisfying strict statistical criteria), or has a mutation that is covered by the expert rule for RRDR (7, 34).

Over time, the reliance on the expert rule will decrease as more evidence regarding individual RRDR mutations becomes available. In fact, WHO is currently conducting the first of a series of regular reviews that are planned as part of the Unitaid-funded Seq&Treat project (5). Importantly, some RRDR mutations might be excluded from the expert rule, should conclusive evidence emerge that these do not confer elevated MICs and/or are not clinically significant. The MIC data from Shea et al. are in line with an earlier report from Haiti by Ocheretina et al. that suggested that *rpoB* T427A may be one of these exceptions (6, 35). However, given that this mutation appears to have evolved independently in lineages 3 and 4, which is typically a signal of selection, additional MIC testing is needed using a method for which a well-defined ECV exists and the quality control range of the laboratory strain H37Rv is not truncated to ensure high-quality testing (17). Moreover, it would be helpful to interrogate whole-genome sequencing data from other collections to see whether T427A has arisen on additional occasions and conduct rigorous MIC testing in those genetic backgrounds.

Limitations of rapid genotypic AST assays. Even though genotypic AST is the preferred approach to detect borderline resistance mutations, these mutations may still go undetected by molecular assays. First, the *rpoB* I491F mutation is not interrogated by any WHO-endorsed assay to date (32). Second, Hain Lifescience launched version 2

of the WHO-endorsed GenoType MTBDR*plus* in 2011. This assay had been designed in a way that missed the borderline L452P mutation, as it was considered to be neutral, whereas version 1 of the assay did detect the mutation. A modified variant of version 2 of the MTBDR*plus* assay was launched in 2014 to reverse this decision, which was followed by an additional update to the assay result interpretation in 2019 (direct communication with Hain Lifescience). Third, *rpoB* L452P was also missed by early versions of the FDA-approved and WHO-endorsed Cepheid Xpert MTB/RIF (Xpert), which prompted changes to the assay (36). Fourth, mutations may be missed if they occur at low frequencies in heteroresistant samples (37).

Conversely, false resistance results are also possible with genotypic AST, which is why FDA has not approved Xpert as a standalone AST assay (i.e., confirmatory testing is mandatory [38]). For example, synonymous RRDR mutations and the aforementioned *rpoB* T427A, should it be truly neutral, cause systematic false resistance results (35, 39). In addition, Xpert has a higher false resistance rate with very low bacillary load samples (37, 40–42). The latter limitation does not apply to Xpert MTB/RIF Ultra, which also was designed to avoid false resistance results due to synonymous mutations at codons 432 and 433 of *rpoB*, although this assay misses some resistance mutations in this region of *rpoB* instead and other synonymous mutations still cause false resistance (37, 39).

Value of next-generation sequencing for discordance analysis. The study by Shea et al. underlines the value of routinely using targeted next-generation sequencing assays or, ideally, whole-genome sequencing not just for cluster analysis but also to investigate discordant or unusual results (5, 6). Indeed, the population structure of MTBC can vary significantly between different settings. For example, Shea et al. reported that most borderline resistant mutants in New York were otherwise pan-susceptible, whereas the *rpoB* I491 clone that now accounts for more than 50% of RIF resistance in Eswatini (formerly known as Swaziland) is resistant to all first-line drugs (32). Moreover, about half of the isolates of this clone have elevated MICs to bedaquiline and clofazimine.

A recent longitudinal study from São Paulo state, which covers 45.5 million inhabitants (i.e., 22% of the Brazilian population), is also very instructive (43). In this setting, 55% (95% confidence interval [CI], 49 to 61%) of genotypically resistant isolates by Xpert tested RIF susceptible at 1 μg/ml using MGIT, which turned out to be due to a combination of three main factors. First, borderline resistance mutations accounted for 61% (95% CI, 53 to 69%) of discordances, largely due to two frequent clusters with *rpoB* H445N. Second, synonymous mutations were responsible for 20% (95% CI, 14 to 27%), again largely due to another prevalent cluster with a synonymous mutation at codon 433. Finally, very low bacillary loads may have been responsible for 6% (95% CI, 3 to 11%) of discordances.

CONCLUSIONS

Even though drug-resistant TB is estimated to account for a quarter of annual deaths attributable to antimicrobial resistance, it is becoming increasingly clear that not following modern principles to assess MICs, PK/PD, and clinical outcome data to set clinical breakpoints has adversely affected patient treatment (8, 44). Indeed, WHO revised numerous incorrect breakpoints for second-line drugs in 2018 and also questioned the validity of all CLSI-endorsed CCs for rifabutin in its aforementioned report (7, 13). We appreciate that the quality and quantity of data in the TB field are limited. However, unless regulators review these data thoroughly and clearly highlight the shortcomings of the available evidence, poorly defined breakpoints will continue to persist in AST guidelines and the scientific literature (15). Indeed, the RIF CC for 7H10 has been used for over half a century despite the lack of supporting evidence. This contributed to the RIF CC for MGIT, the most widely used commercial phenotypic AST method, being set above the tentative ECV. In addition, this problem was particularly marked for the now-discontinued Bactec 460 system by Becton Dickinson and likely extends to other assays, including 7H11, microscopic observation direct susceptibility testing, MYCOTB, and VersaTREK (6, 7, 25, 45).

EUCAST has recently endorsed a reference method for which it will set clinical breakpoints in the future (16). Moreover, it has developed a standard operating

procedure for calibrating other methods against this reference, as is standard practice for other bacterial pathogens. This approach will almost certainly have to be refined based on the experience gained by calibrating the first set of drugs but nonetheless represents the best approach for setting sound breakpoints and validating alternative methods. We call upon the wider TB community, including CLSI, FDA, WHO, funders, industry, researchers, and global policymakers, to engage with this process to ensure more robust guidance (44). In particular, there is an urgent need for the development and rigorous evaluation of a commercial 96-well microdilution plate to enable high-quality, routine MIC testing for traditional as well as newly approved anti-TB agents.

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