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Are Current Critical Concentrations for Mycobacterium tuberculosis Drug Susceptibility Testing in Egg-Based Media Adequate? Insights from Phenotypic and Genotypic Analyses

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Abstract

Phenotypic antimicrobial susceptibility testing (pAST) remains the reference standard for detecting drug resistance in *Mycobacterium tuberculosis* (Mtb), particularly for identifying resistance levels that cannot be inferred by molecular methods alone. This study evaluated the performance of the Ogawa-Kudoh (O-K) egg-based medium for minimum inhibitory concentration (MIC) determination and establishment of epidemiological cutoff values (ECOFFs) for first- and second-line anti-tuberculosis drugs. A total of 65 Mtb strains with diverse resistance profiles were tested against isoniazid (INH), rifampicin (RIF), levofloxacin (LFX), moxifloxacin (MFX), linezolid (LZD), bedaquiline (BDQ), and pretomanid (Pa). MICs obtained in O-K medium were compared with the EUCAST broth microdilution reference method and genotypic assays. ECOFFs determined in O-K medium were 0.2 mg/L for INH, 20 mg/L for RIF, 1 mg/L for LFX, MFX, and LZD, and 4 mg/L for Pa. Agreement between O-K and the EUCAST reference method was almost perfect ($\kappa = 0.983$; 95% CI: 0.959–1.000), with only two discordant results among 356 MIC determinations. Phenotypic methods showed excellent concordance ($\kappa > 0.9$), whereas agreement between phenotypic and genotypic approaches was lower ($\kappa < 0.8$), largely due to heteroresistance, incomplete mutation coverage, and mutations not associated with clinically relevant resistance. Minimal overlap between phenotypically wild-type and non-wild-type MIC distributions were observed, except for moxifloxacin within the technical uncertainty range. These findings support the use of O-K medium as a reliable and accessible platform for phenotypic susceptibility testing of Mtb, particularly in resource-limited settings.

1. INTRODUCTION

The World Health Organization (WHO) recommends molecular antimicrobial susceptibility testing (mAST) as the initial approach to detect resistance in all tuberculosis (TB) patients, including both new and retreatment cases [1]. Genotypic methods, such as next-generation sequencing, offer significant advantages over culture-based drug susceptibility testing (DST), including faster turnaround times and the ability to analyze multiple resistance-associated genetic regions simultaneously [1, 2].

Despite these advantages, molecular diagnostics present important limitations. Their mutation coverage is incomplete, and they do not quantify resistance levels. Specifically, genotypic tests detect the presence or absence of mutations but do not measure the minimum inhibitory concentration (MIC), which is essential to distinguish between high-level resistance and mutations associated with modest MIC increases [1, 3, 4]. As a result, discrepancies between genotypic and phenotypic methods are common. Some isolates harbor resistance-associated mutations but remain phenotypically susceptible, while others lack detectable mutations yet display phenotypic resistance. In addition, low-level resistance mutations—such as certain variants in the inhA promoter or other genes—may confer moderate resistance or cross-resistance, often resulting in borderline or intermediate phenotypic profiles and apparent inconsistencies between methods [5].

In this context, phenotypic antimicrobial susceptibility testing (pAST) remains the gold standard for detecting drug resistance in *Mycobacterium tuberculosis* (Mtb) [2]. Traditionally, pAST relies on a single critical concentration (CC) to differentiate susceptible from resistant isolates, with values specific to each drug and testing method. In 2017 and 2020, systematic reviews were conducted to revise CCs for first- and second-line anti-TB drugs, leading to updated recommendations that better reflect current knowledge of resistance mechanisms [6, 7]. However, the available MIC data remain limited, particularly for egg-based media, highlighting the need for further evaluation of CCs across different testing platforms [6, 7].

Given these gaps, this study aimed to determine epidemiological cutoff values (ECOFFs) for first- and second-line anti-TB drugs using an egg-based medium, and to compare these results with genotypic methods, with a particular focus on low-level resistance variants associated with modest MIC increases. The Ogawa-Kudoh (O-K) medium was selected due to its well-established use and because it forms the basis of the Swab Culture – Optimized Test (SCOT-TB), a dip-slide culture system recently developed by our group. SCOT-TB comprises four Ogawa medium surfaces and, when used in accordance with the O-K method, enables the simultaneous detection of the Mtb complex and the assessment of drug susceptibility, offering a practical and potentially scalable approach for TB diagnosis and resistance testing.

2. MATERIALS AND METHODS

2.1 Study design

We evaluated the minimum inhibitory concentrations (MICs) of isoniazid (INH), rifampicin (RIF), levofloxacin (LFX), moxifloxacin (MFX), bedaquiline (BDQ), linezolid (LZD), and pretomanid (Pa) against 65 Mtb strains with diverse resistance profiles. Testing was performed using O-K medium and compared to the reference broth microdilution method in Middlebrook 7H9 supplemented with 10% oleic acid–albumin–dextrose–catalase (OADC), aligned with EUCAST guidelines, as well as selected commercial assays [8, 9]. A flowchart summarizing the laboratory procedures is presented in Fig. 1.

MIC: minimum inhibitory concentration; MGIT: Mycobacteria Growth Indicator Tube; SIRE: streptomycin, isoniazid, rifampicin and ethambutol; MTB: *Mycobacterium tuberculosis*; RIF: rifampicin; Image created with flaticon.com.

2.2 Antibiotic susceptibility testing

Antimicrobials (INH, RIF, LFX, MFX, LZD, Pa) were obtained from Sigma-Aldrich and BDQ from Santa Cruz Biotechnology. Stock solutions were prepared according to CLSI guidelines and stored at − 80°C. The strain panel included 61 clinical isolates and four reference strains (Mtb H37Ra ATCC®25177™, H37Rv ATCC®27294™, H37Rv-INH-R ATCC®35822™, and H37Rv-RIF-R ATCC®35838™) from the Mycobacteriology Laboratory at NDI/UFES.

For both methods, frozen cultures were thawed and grown on OK slants at 37°C for up to four weeks. Two loopfuls of colonies were transferred to tubes containing glass beads and 0.2 ml sterile deionized water, vortexed, allowed to settle, diluted with 3 ml SDW, and vortexed again. After settling, 0.5 ml of the supernatant was mixed with 4.5 ml SDW. The suspension was adjusted spectrophotometrically (625 nm) to an absorbance of 0.500–0.550, corresponding to approximately 10^7 CFU ml^{-1}. Serial dilutions of 10^{-2} and 10^{-4} were prepared in Middlebrook 7H9 with OADC.

The protocol for MIC in Ogawa-Kudoh was adapted from the agar dilution method. Six concentrations per drug were incorporated into the medium before inspissation [10]: 1–0.05 mg l^{-1} (INH), 80–5 mg l^{-1} (RIF), 8–0.25 mg l^{-1} (LFX and Pa), 4–0.12 mg l^{-1} (MFX and LZD), and 1–0.03 mg l^{-1} (BDQ). Plates were inspissated at 85°C for 60 min and stored at 4°C for up to three days. For inoculation, 0.1 ml of the 10^{-2} dilution was added to drug-containing and control quadrants, while three drops (0.01 ml each) of the 10^{-4} dilution were added to a second control quadrant for colony counting. Plates were incubated at 37°C and read after three weeks. Validity required the 10^{-2} inoculum to correspond to 5×10^4–5×10^5 CFU ml^{-1}. MIC was defined as the lowest concentration preventing visible growth.

The EUCAST reference method used 96-well microtiter plates with drug concentrations ranging from 0.4–0.003 mg l^{-1} (INH), 2–0.016 mg l^{-1} (RIF and Pa), 4–0.03 mg l^{-1} (LFX, LZD, BDQ), and 1–0.008 mg l^{-1} (MFX) [3,4,27]. Plates were stored at − 20°C for up to three days. At inoculation, 0.1 ml of the 10^{-2} suspension was added to drug-containing wells and 100% growth control, and 0.1 ml of the 10^{-4} dilution to 1% growth control wells. Plates were sealed in CO_2-permeable bags, incubated at 37°C, and read after 14 days. Tests were valid if growth occurred in the 1% control and inoculum density was within the acceptable range. MIC was defined as the lowest concentration inhibiting visible growth, with EUCAST breakpoints applied: 0.1 mg l^{-1} (INH), 0.25 mg l^{-1} (MFX), 0.5 mg l^{-1} (RIF and Pa), and 1 mg l^{-1} (LFX, LZD, BDQ).

The BD BACTEC™ MGIT™ SIRE assay was performed following manufacturer instructions. Five tubes were used: one growth control inoculated with the 10^{-4} dilution, and four tubes containing streptomycin, INH, RIF, or ethambutol inoculated with the 10^{-2} dilution. Tubes were incubated in the MGIT 960 system, which automatically interpreted results as susceptible or resistant.

2.3 Genetic characterization

Xpert® MTB/RIF Ultra (Cepheid) was conducted as previously described [33]. Briefly, 1.5 ml of the 10^{-2} inoculum was mixed with 3 ml of sample reagent, incubated for 15 min, and 2 ml transferred to the cartridge for automated processing. Results were reported as Mtb detected or not detected, and rifampicin resistance detected or not detected.

Line-probe assays (GenoType MTBDRplus v2.0 and MTBDRsl v2.0, Hain Lifescience) were performed using DNA extracted with the GenoLyse kit. A loopful of bacterial growth was processed with lysis buffer, heat treatment, and neutralization, followed by centrifugation. The supernatant containing DNA was stored at − 80°C. For amplification, master mixes were prepared in a pathogen-free environment, and DNA was added separately. PCR was performed in an Applied Biosystems thermocycler using protocols for cultured samples. Amplified products underwent strip hybridization following the manufacturer's instructions. DNA regions covered by the probes used in the genotypic assays are shown in Fig. 2.

E. coli: *Escherichia coli*; Mtb: *Mycobacterium tuberculosis*; WT: wild type; RIF: rifampicin; bp: base pairs; *rpoB*: DNA-directed RNA polymerase subunit beta gene; *fabG1*: 3-oxoacyl-ACP reductase FabG gene; *inhA*: NADH-dependent enoyl-[ACP] reductase gene; *katG*: catalase-peroxidase gene; *gyrA*: DNA gyrase subunit A gene; *gyrB*: DNA gyrase subunit B gene; Red blocks: corresponding codons of the known mutation probes from MTBDR*plus* and MTBDR*sl*; Gray blocks: wild type probes from MTBDR*plus* and MTBDR*sl*; Blue blocks: wild type probes from Xpert MTB/RIF Ultra.

Results were interpreted based on the presence or absence of specific hybridization bands. Agreement between phenotypic and genotypic methods was assessed using Cohen's kappa coefficient and interpreted according to McHugh criteria.

2.4 Statistical analysis and ethical aspects

Agreement between tests was calculated using Cohen's kappa and classified as suggested by McHugh [11]. The study was approved by the Research Ethics Committee of the Centro de Ciências da Saúde at UFES (Protocol 65501122.9.0000.5060).

3. RESULTS

The results of phenotypic and genotypic assays are presented in Supplemental Tables SM1 and SM2. The EUCAST reference method was used to classify strains as phenotypically wild type (pWT) when MIC values were below the breakpoint, or phenotypically non-wild type (pNWT) when above. Similarly, GenoType MTBDRplus and MTBDRsl assays classified strains as genotypically wild type (gWT) in the absence of mutations, or genotypically non-wild type (gNWT) when mutations were detected. Susceptibility profiles for rifampicin (RIF) and isoniazid (INH) are summarized in Tables 1 and 2, while those for levofloxacin (LFX), moxifloxacin (MFX), linezolid (LZD), pretomanid (Pa), and bedaquiline (BDQ) are shown in Supplementary Tables SM3–SM7.

3.1 Rifampicin

For RIF, all assays agreed on 24 resistant and 23 susceptible strains, while 18 strains showed discordant results. MGIT identified 29 resistant and 36 susceptible strains. In contrast, the EUCAST reference method classified 31 strains as pNWT and 34 as pWT (MIC ≤ 0.5 mg/L). These two phenotypic methods agreed on 63 strains but differed for CI-35 and CI-43. Both strains were susceptible by MGIT but had MIC values above the breakpoint in Middlebrook 7H9 (2 and 1 mg/L, respectively) and very high MICs in O-K (40 and 80 mg/L). Both were classified as gNWT. Additionally, CI-58 showed a very high MIC in O-K (> 80 mg/L) despite being susceptible in MGIT and borderline (0.5 mg/L) in 7H9.

Genotypically, Xpert MTB/RIF Ultra detected rpoB mutations in 37 strains, no mutations in 27, and one indeterminate result (CI-03). MTBDRplus identified mutations in 39 strains and none in 26. Known mutations included D516V, H526D, H526Y, and S531L, the latter being most frequent. Non-specific mutations were also observed across several codon regions. The two genotypic methods agreed on 61 strains (36 gNWT and 25 gWT), but differed for four (CI-03, CI-15, CI-58, CI-59). CI-15 and CI-59 exhibited heteroresistance in MTBDRplus but were classified as susceptible by Xpert. CI-03 yielded an indeterminate result in Xpert but showed a non-wild-type pattern in MTBDRplus. These strains were phenotypically resistant with high MICs. CI-58 showed discordance across both phenotypic and genotypic assays. Further discrepancies were observed when comparing phenotypic and genotypic methods. Two strains (CI-33 and CI-34) were phenotypically resistant but lacked detectable mutations. Conversely, ten pWT strains exhibited non-specific rpoB mutations.

3.2 Isoniazid

For INH, all assays agreed on 30 resistant and 30 susceptible strains, with discrepancies in five cases. MGIT reported 35 resistant and 30 susceptible strains, whereas EUCAST classified 34 as pNWT and 31 as pWT (MIC ≤ 0.1 mg/L). The only phenotypically discordant strain, CI-56, was resistant in MGIT but had a borderline MIC (0.1 mg/L) in 7H9 and 0.2 mg/L in O-K. It was classified as gWT. MTBDRplus identified 35 gWT and 30 gNWT strains. Mutations included katG S315T (most frequent), inhA promoter mutations (notably c-15t), katG deletions, and combined mutations. Some strains showed heteroresistance. Notably, four strains (CI-17, CI-25, CI-46, CI-58) showed no mutations but were phenotypically resistant with high MIC values across media, indicating possible resistance mechanisms not captured by the assay.

3.3 Levofloxacin

For LFX, all methods agreed on 60 susceptible and 3 resistant strains, with discrepancies in two cases. EUCAST classified 62 strains as pWT (MIC ≤ 1 mg/L) and 3 as pNWT. MICs for pWT ranged from 0.12–0.5 mg/L in 7H9 and ≤ 0.25–1 mg/L in O-K, while pNWT strains had higher MICs. MTBDRsl identified 60 gWT and 5 gNWT strains. Known mutations included gyrA A90V and D94G, all associated with pNWT strains. Two strains with non-specific gyrA mutations (CI-16, CI-61) were phenotypically susceptible with low MICs. No gyrB mutations were detected.

3.4 Moxifloxacin

For MFX, agreement was observed for 59 susceptible and 3 resistant strains, with three discordant cases. EUCAST classified 61 strains as pWT (MIC ≤ 0.25 mg/L) and 4 as pNWT. MIC ranges were similar to LFX but slightly lower. CI-43 was classified as pNWT by EUCAST (MIC 0.5 mg/L) but as pWT by MTBDRsl, with an MIC of 1 mg/L in Ogawa-Kudoh. This value overlapped with pWT strains, creating a zone of uncertainty. Genotypic results mirrored those of LFX, with the same gyrA mutations detected. Strains with non-specific mutations remained phenotypically susceptible. No gyrB mutations were identified.

3.5 Linezolid

All 65 strains were classified as pWT for LZD by EUCAST (MIC ≤ 1 mg/L). MIC values ranged from 0.12 to 1 mg/L in 7H9 and 0.25 to 1 mg/L in O-K. No genotypic testing was performed.

3.6 Pretomanid

A total of 31 strains were tested for Pa. EUCAST classified 29 as pWT (MIC ≤ 0.5 mg/L) and 2 (CI-38 and CI-52) as pNWT. pWT MICs ranged from ≤ 0.016 to 0.12 mg/L in 7H9 and 0.5 to 4 mg/L in O-K. Resistant strains had MICs > 2 mg/L in liquid and > 8 mg/L in solid medium. No genotypic assays were conducted.

3.7 Bedaquiline

For BDQ, 38 strains were evaluated using the EUCAST method, all classified as pWT (MIC ≤ 1 mg/L), with MICs ranging from ≤ 0.03 to 0.5 mg/L. Only 12 strains were tested in O-K, all showing MIC > 1 mg/L. Due to limited data, no genotypic analysis or robust conclusions could be drawn.

3.8 ECOFF in Ogawa-Kudoh

The epidemiological cut-off values (ECOFFs) in O-K medium were determined as follows: 20 mg/L for RIF, 0.2 mg/L for INH, 4 mg/L for Pa, and 1 mg/L for LFX, MFX, and LZD (Fig. 3). Due to insufficient data, no ECOFF could be established for BDQ. Overall, MIC distributions for pWT and pNWT strains did not overlap, with two exceptions. For MFX, CI-43 overlapped with several pWT strains at 1 mg/L, indicating a technical uncertainty zone. Conversely, CI-58, a pWT strain, showed an unusually high MIC for RIF (> 80 mg/L) in O-K. A total of 356 MIC assays were performed in each medium (excluding BDQ and repeat tests). Agreement between O-K and the EUCAST reference method was almost perfect (Cohen's $\kappa = 0.983$; 95% CI: 0.959–1.000), with only two discordant results. Phenotypic methods showed excellent agreement ($\kappa > 0.9$), whereas agreement between phenotypic and genotypic methods was lower ($\kappa < 0.8$), reflecting inherent methodological differences.

MIC: minimum inhibitory concentration; RIF: rifampicin; INH: isoniazid; LFX: levofloxacin; MFX: moxifloxacin; LZD: linezolid; Pa: pretomanid; pWT: phenotypically wild type; pNWT: phenotypically non-wild type; ECOFF: epidemiological cut-off value; ATU: area of technical uncertainty; Blue bars: pWT strains based on EUCAST reference method results; Red bars: pNWT strains based on EUCAST reference method results; Green line: ECOFF based on MIC in Ogawa-Kudoh results; Yellow area: ATU based on overlapping of pWT and pNWT results.

4. DISCUSSION

4.1 Rifampicin

Drug-resistant tuberculosis (DR-TB) remains a major global public health concern and is listed among WHO priority pathogens [12]. Resistance to rifampicin (RIF), the most potent first-line drug, is particularly critical because it defines rifampicin-resistant TB (RR-TB) and serves as a proxy for multidrug-resistant TB (MDR-TB), both requiring second-line treatment regimens [13]. Despite advances in diagnostic tools, DR-TB remains underdiagnosed, contributing to ongoing transmission and poor treatment outcomes.

A key factor contributing to underdiagnosis is the suboptimal growth of certain RR-TB strains in liquid culture systems, which may lead to false susceptibility results [14, 15]. Previous studies have reported discordances between Löwenstein-Jensen (LJ) and MGIT systems, especially in strains harboring specific rpoB mutations such as L511P, D516Y, L533P, I572F, and alterations at codon H526 [3]. In these cases, MGIT may fail to detect resistance or misclassify isolates as susceptible, whereas solid media such as LJ tend to provide more reliable detection. This likely explains why MGIT failed to identify resistance in strains CI-35 and CI-43, and why strain CI-58 showed high MIC values in the egg-based O-K medium but appeared susceptible in liquid assays.

Heteroresistance further contributes to discordant results [16, 17]. This condition occurs when susceptible and resistant subpopulations coexist within the same isolate. Phenotypic antimicrobial susceptibility testing (pAST) can generally detect resistance when at least 1% of the population is resistant, making it suitable for identifying heteroresistance. In contrast, genotypic methods (gAST), which rely on detecting specific mutations, may fail if wild-type sequences are still present. Although Xpert MTB/RIF Ultra has improved sensitivity for detecting heteroresistance based on melting temperature differences, it failed to identify heteroresistance in strains CI-15 (rpoB D516V) and CI-59 (rpoB H526Y), which were detected by MTBDRplus [18]. Conversely, Ultra may have detected heteroresistance in CI-58 that MTBDRplus missed [18]. Additionally, mutations such as L511P and H526N, not targeted by MTBDRplus probes, have been associated with discordant phenotypic profiles, often appearing susceptible in liquid media but resistant in solid media [3]. So, we hypothesize that all the phenotypic and genotypic discordances of CI-58 may be due to a heteroresistance caused by Mtb strains with *rpoB* L511P or H526N.

Further discrepancies between phenotypic and genotypic results may arise from mutations outside the rifampicin resistance-determining region (RRDR). For example, strains CI-33 and CI-34 may harbor mutations such as V170F or I491F, which confer resistance but are not detected by standard molecular assays [1]. Conversely, several isolates carrying non-specific RRDR mutations (e.g., CI-07, CI-12, CI-19, CI-21, CI-23, CI-48, CI-49, CI-51, CI-55, CI-57) were phenotypically susceptible, indicating that not all detected mutations result in clinically relevant resistance [1].

4.2 Isoniazid

The WHO catalogue of resistance-associated mutations for isoniazid (INH) identifies a limited set of key genetic alterations, mainly involving promoter mutations in the fabG1-inhA operon and mutations in the katG gene [1]. Mutations in inhA are typically associated with low-level resistance, whereas katG mutations confer high-level resistance [1]. The MTBDRplus assay targets most common mutations but does not cover all relevant regions, such as inhA g-154a and katG W328L. This incomplete coverage may explain why strains CI-17, CI-25, CI-46, and CI-58 were classified as genotypically wild type despite elevated MIC values in phenotypic assays.

These findings highlight a key limitation of current molecular diagnostics: incomplete mutation coverage. Another example of discordance is strain CI-56, classified as resistant by MGIT but showing borderline MIC values in both liquid and solid media and a wild-type genotype. Although some INH-resistant strains may grow better in liquid media, this explanation is unlikely here, as the EUCAST liquid method showed agreement with solid media results [19]. The discrepancy is more plausibly attributed to inherent variability in phenotypic testing, particularly for isolates with MIC values close to the critical concentration.

4.3 Fluoroquinolones

Fluoroquinolones such as levofloxacin (LFX) and moxifloxacin (MFX) are key components of DR-TB treatment regimens. Resistance is mainly associated with mutations in the gyrA and gyrB genes [1]. The MTBDRsl assay targets critical regions of these genes, but not all detected mutations are clinically relevant. For example, mutations such as gyrA A90G and S95T are not associated with resistance but may disrupt hybridization of wild-type probes, leading to false classification as resistant [1]. This likely explains the findings for strains CI-16 and CI-61, which showed genotypic alterations but remained phenotypically susceptible with low MIC values. An important observation was the overlap between susceptible and resistant MIC distributions for MFX in the O-K medium, typical of low-level resistance where MIC values fall within a borderline range. In such cases, repeated phenotypic testing may yield variable results. To address this issue, EUCAST introduced the concept of areas of technical uncertainty (ATU), defining MIC ranges where interpretation is unreliable [20]. For MFX, MIC values of 0.25 mg/L in liquid media or 1 mg/L in O-K fall within this uncertainty zone. Strain CI-43 exemplifies this situation: although classified as resistant by both EUCAST and MTBDRsl, its MIC in O-K fell within the ATU range. This discrepancy is likely due to methodological variability rather than true disagreement [4, 20].

4.4 Pretomanid

Pretomanid (Pa) is a relatively new drug introduced for MDR/RR-TB treatment [21]. Despite its recent use, resistance has already been identified in strains from treatment-naive patients [3, 15, 22]. Two main mechanisms have been described: intrinsic low-level resistance associated with specific lineages and acquired high-level resistance due to mutations in the ddn gene [3, 15, 22].

In this study, strains CI-38 and CI-52 exhibited very high MIC values (> 8 mg/L), strongly suggesting ddn mutation s. Notably, these strains were isolated prior to the clinical introduction of pretomanid, indicating that resistance-conferring mutations may already be circulating. This finding underscores the importance of implementing robust antimicrobial susceptibility testing (AST) methods for pretomanid in both clinical management and epidemiological surveillance [22]. Early resistance emergence could compromise the long-term effectiveness of newer regimens such as BPaL(M).

4.5 Bedaquiline

Unlike other drugs evaluated, no established critical concentration exists for bedaquiline (BDQ) in egg-based media such as LJ. Initial testing used concentration ranges based on 7H11 agar standards. However, all strains grew at the highest BDQ concentration in the O-K medium, despite being classified as susceptible in liquid assays. This discrepancy can be explained by the influence of protein content in culture media. Increased protein levels, such as those in egg-based media or with bovine serum albumin (BSA), can significantly elevate BDQ MIC values [23]. As a result, BDQ appears less active in these media compared to standard agar or liquid systems. Given these limitations, and considering the parallel evaluation of pretomanid, further BDQ testing was deprioritized. These findings highlight the challenges in standardizing AST for BDQ and emphasize the need for further research to establish reliable testing conditions.

4.6 ECOFF in Ogawa-Kudoh

Egg-based media have long been used for TB diagnostics, particularly in Japan, where Ogawa medium is widely employed [24]. The O-K medium, although slightly different in composition and buffering capacity, has demonstrated comparable performance [24]. In this study, epidemiological cutoff values (ECOFFs) determined for O-K were consistent with WHO-recommended critical concentrations for LJ for several drugs, including INH, MFX, and linezolid (LZD) [6, 7]. These results support the potential use of O-K as an alternative medium for phenotypic AST. However, some discrepancies were observed. For INH, the CLSI-recommended critical concentration for LJ (0.25 mg/L) may be too high, potentially leading to misclassification of low-level resistant strains as susceptible. For RIF, WHO currently recommends maintaining a higher critical concentration due to limited data, but these findings suggest that a lower value (≤ 20 mg/L) may be more appropriate, pending further validation. For LFX, the ECOFF in O-K was slightly lower than previously reported values, possibly due to the limited number of resistant isolates [25]. Additional studies with larger sample sizes are needed to refine these thresholds.

Overall, minimal overlap between phenotypically wild-type (pWT) and non-wild-type (pNWT) MIC distributions was observed, indicating good discriminatory capacity of the medium. The few exceptions likely reflect biological variability or borderline resistance mechanisms. Importantly, agreement between O-K and the EUCAST reference method was nearly perfect, supporting the reliability of this medium for phenotypic testing.

5. CONCLUSION

This study demonstrates that antimicrobial susceptibility testing of *M. tuberculosis* using the O-K medium provides satisfactory performance for detecting resistance to rifampicin (RIF), isoniazid (INH), levofloxacin (LFX), moxifloxacin (MFX), linezolid (LZD), and pretomanid (Pa), but not for bedaquiline (BDQ). The ECOFF for INH was consistent with the critical concentration used in LJ (0.2 mg/L), while the ECOFF for RIF (20 mg/L) suggests that current critical concentrations in egg-based media may require revision, particularly for detecting low-level resistance. LFX, MFX, LZD, and Pa showed good compatibility with the medium, whereas BDQ demonstrated reduced activity. The three phenotypic testing methods showed near-perfect agreement, although concordance decreased when compared with genotypic assays. Non-specific mutations detected by molecular methods were not consistently associated with increased MIC values, reinforcing the need for cautious interpretation of genotypic results. Overall, O-K represents a reliable and accessible alternative for phenotypic drug susceptibility testing, particularly in resource-limited settings.

Declarations

Authors Contributions: All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Luiz Guilherme Schmidt Castellani, Manuela Negrelli Brunetti, Brunelli da Rós Peruch Monier, Gérson Fontana and Ana Karolina de Alvarenga Valim. The first draft of the manuscript was written by Luiz Guilherme Schmidt Castellani and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Figures

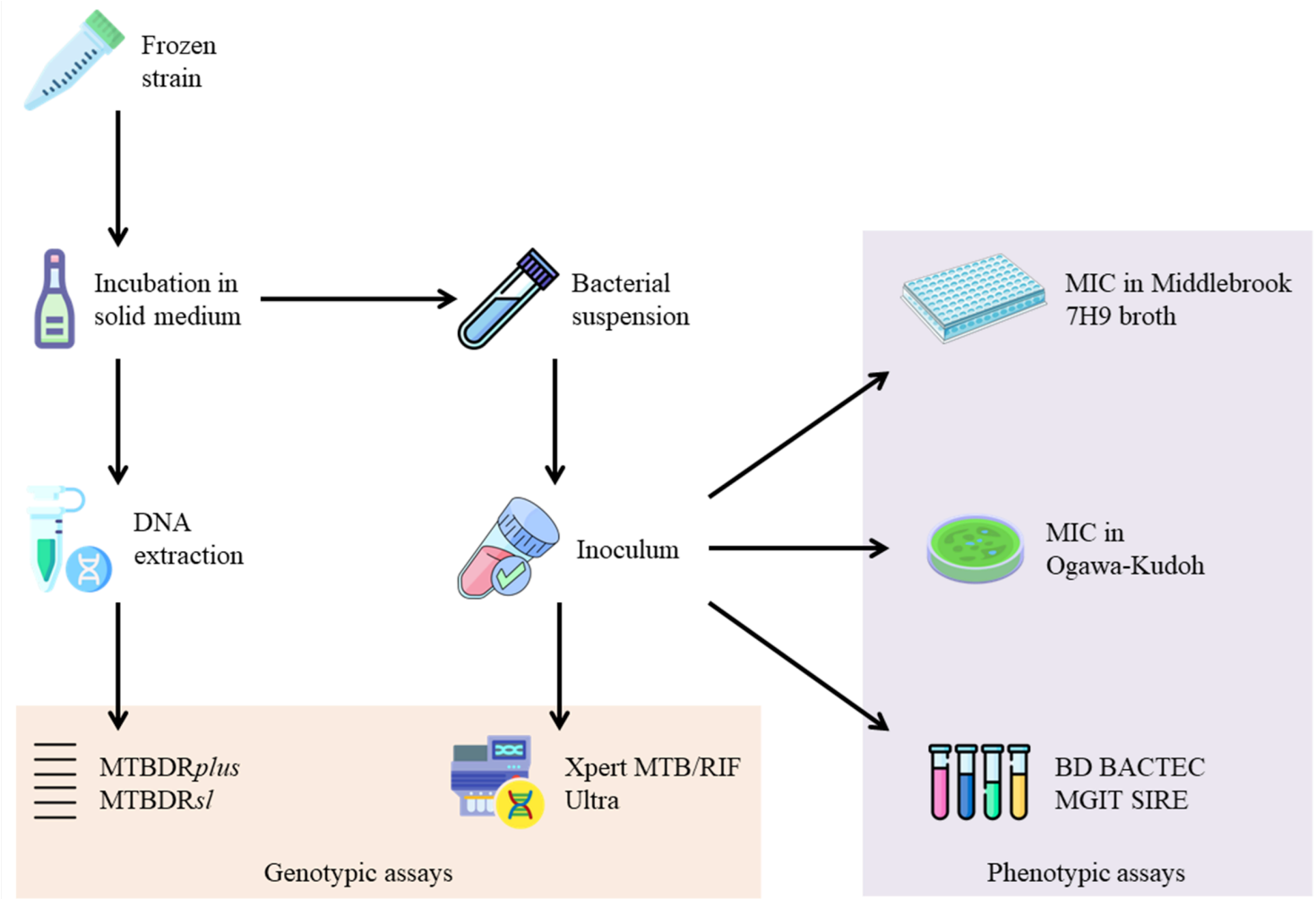


Figure 1

Flowchart of the laboratory procedures.

MIC: minimum inhibitory concentration; MGIT: Mycobacteria Growth Indicator Tube; SIRE: streptomycin, isoniazid, rifampicin and ethambutol; MTB: *Mycobacterium tuberculosis*; RIF: rifampicin; Image created with flaticon.com.

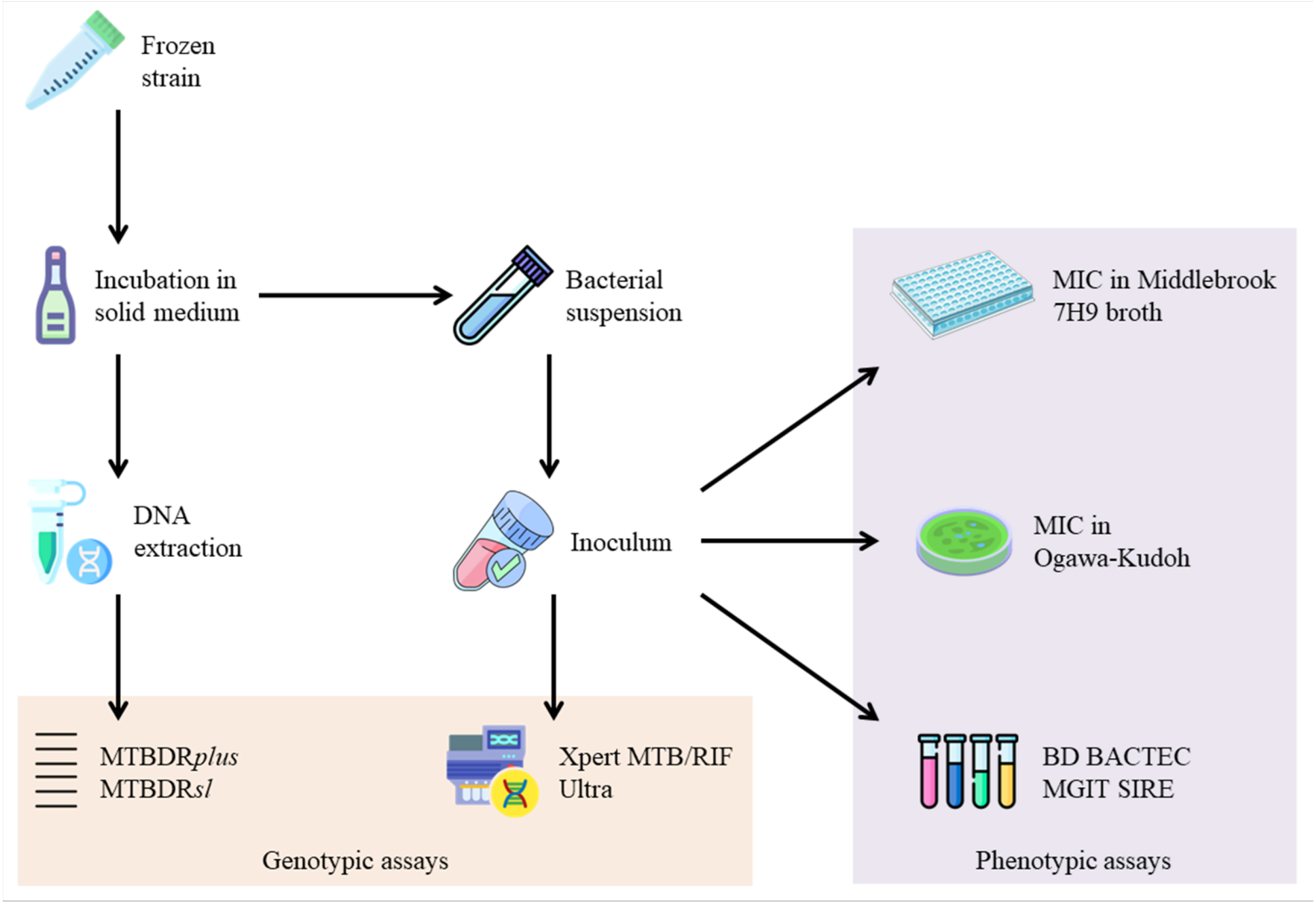


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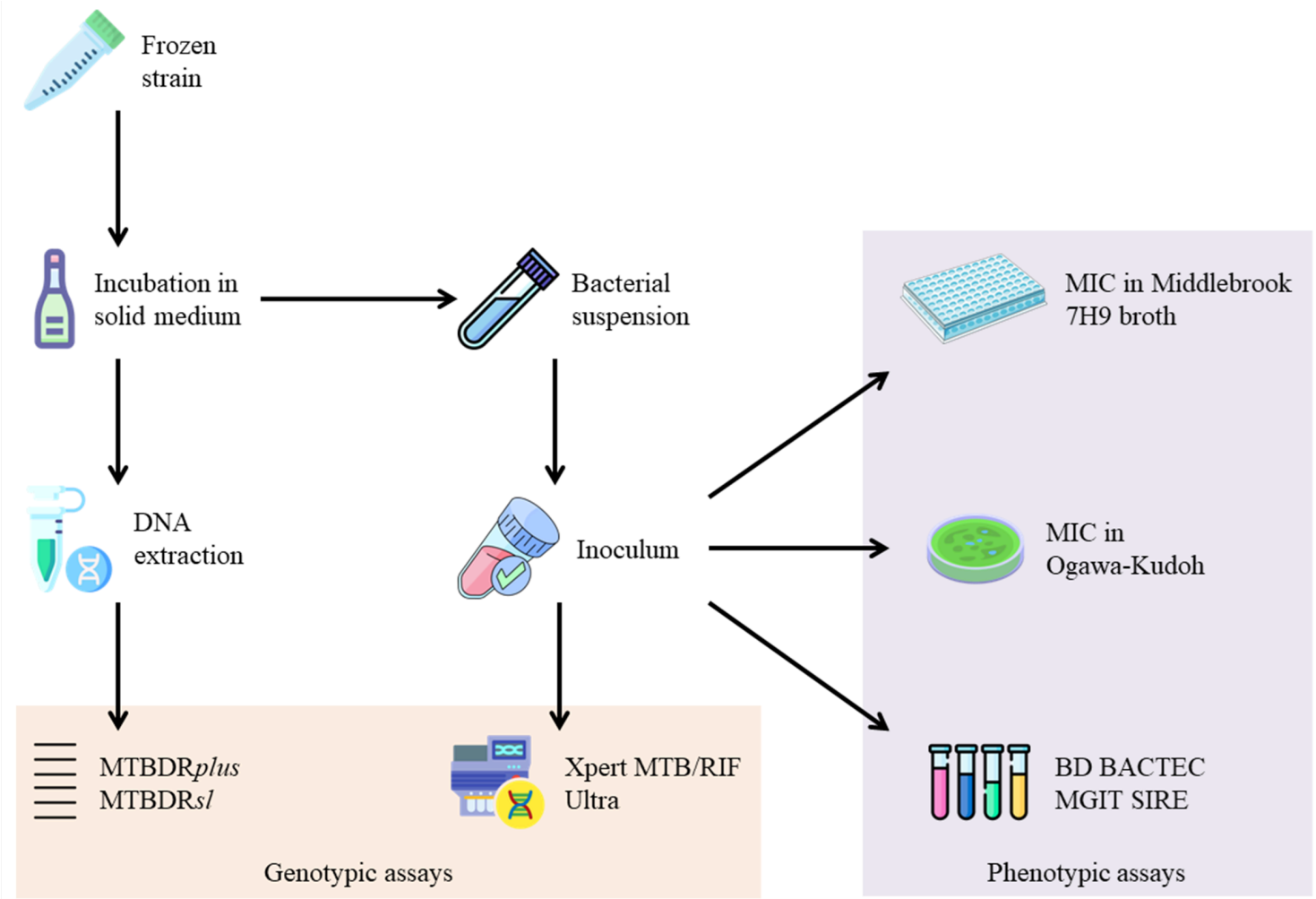


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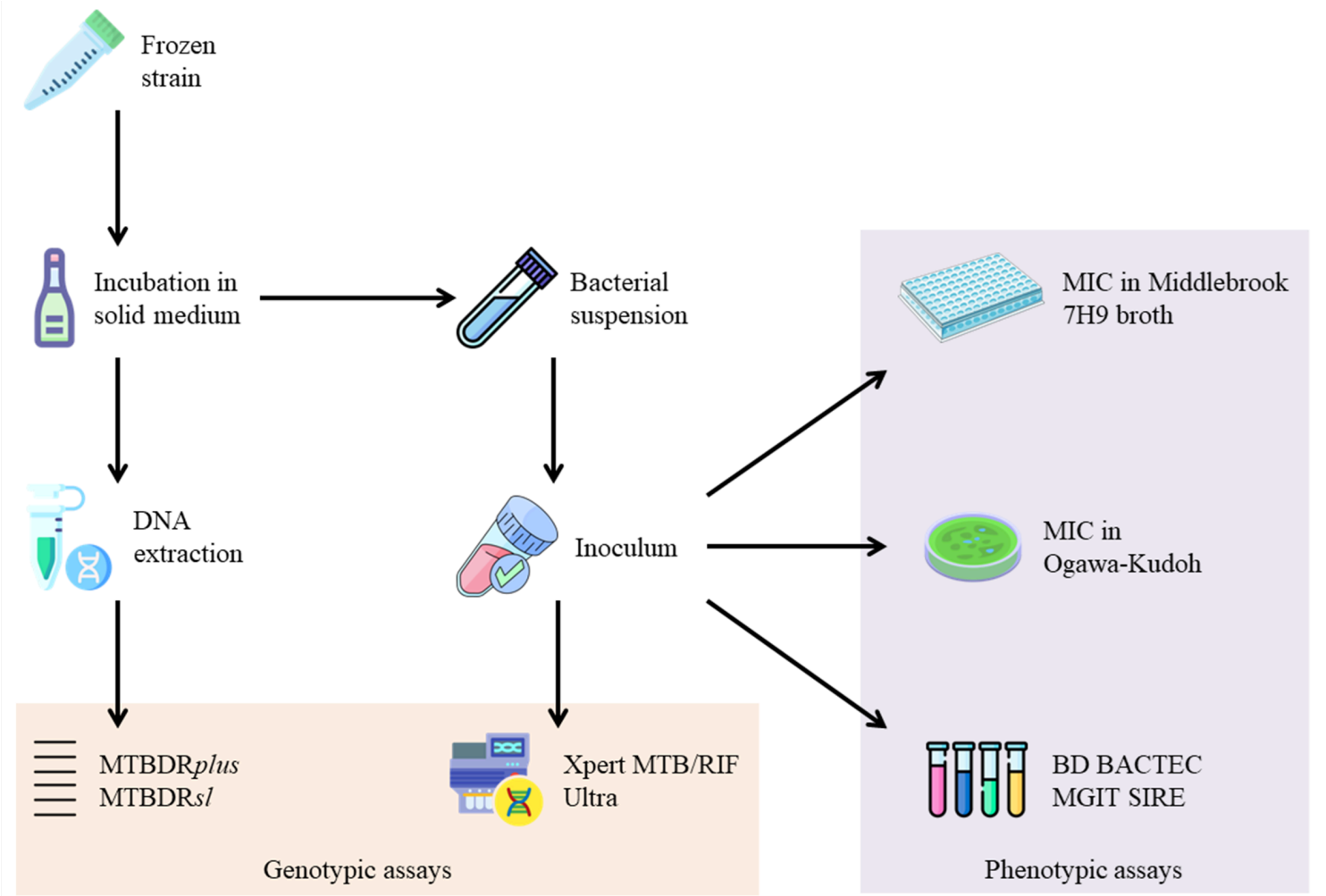


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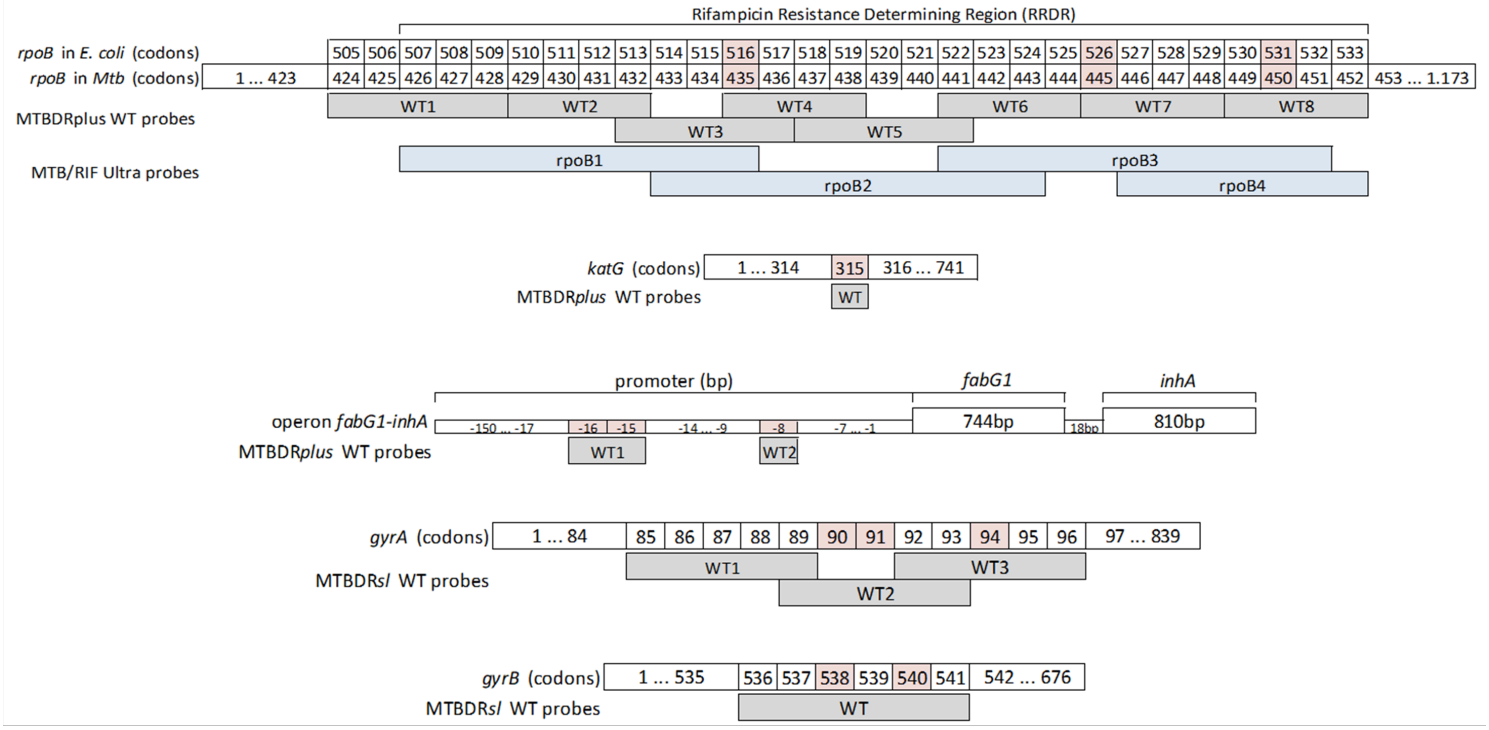


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DNA coverage of the genotypic assays.

E. coli: *Escherichia coli*; Mtb: *Mycobacterium tuberculosis*; WT: wild type; RIF: rifampicin; bp: base pairs; *rpoB*: DNA-directed RNA polymerase subunit beta gene; *fabG1*: 3-oxoacyl-ACP reductase FabG gene; *inhA*: NADH-dependent enoyl-[ACP] reductase gene; *katG*: catalase-peroxidase gene; *gyrA*: DNA gyrase subunit A gene; *gyrB*: DNA gyrase subunit B gene; Red blocks: corresponding codons of the known mutation probes from MTBDR*plus* and MTBDR*sl*; Gray blocks: wild type probes from MTBDR*plus* and MTBDR*sl*; Blue blocks: wild type probes from Xpert MTB/RIF Ultra.

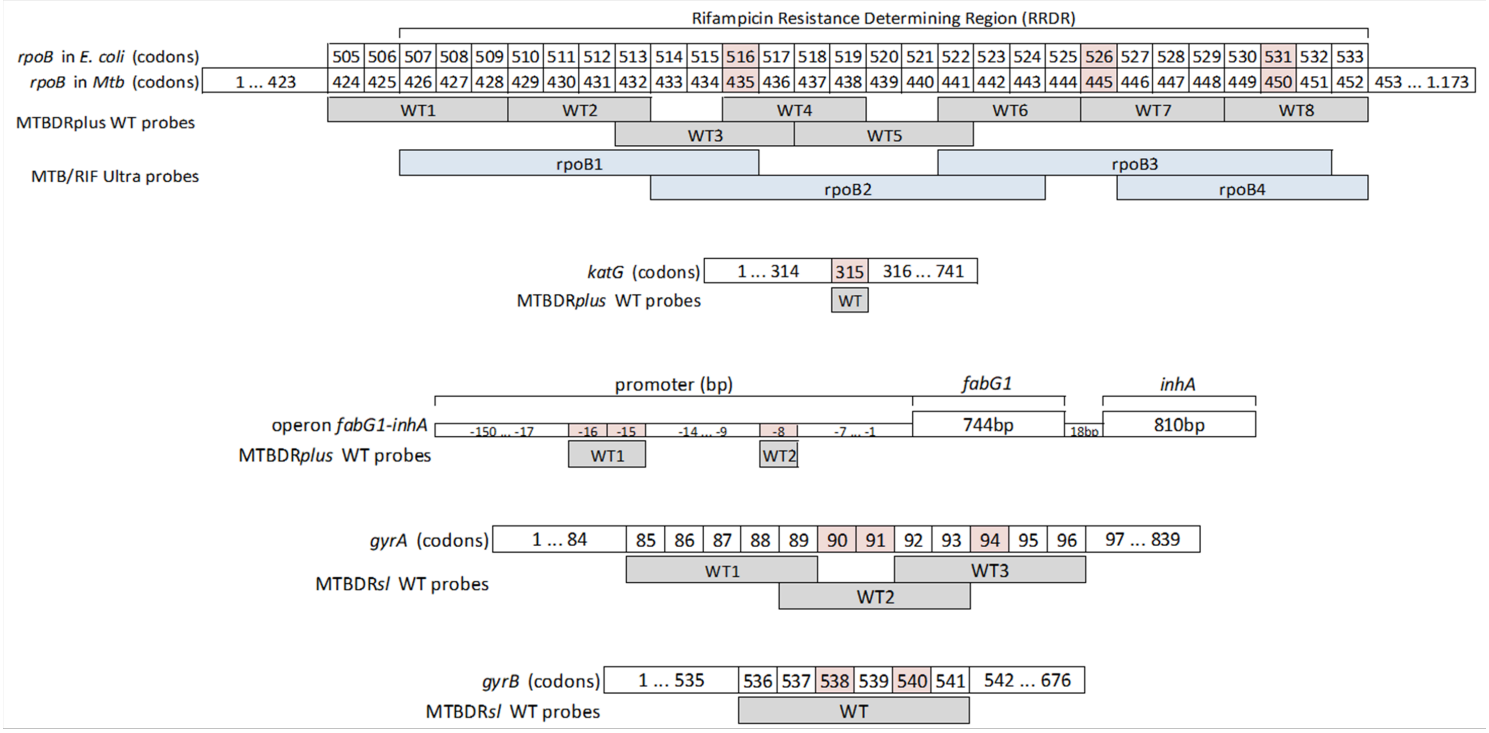


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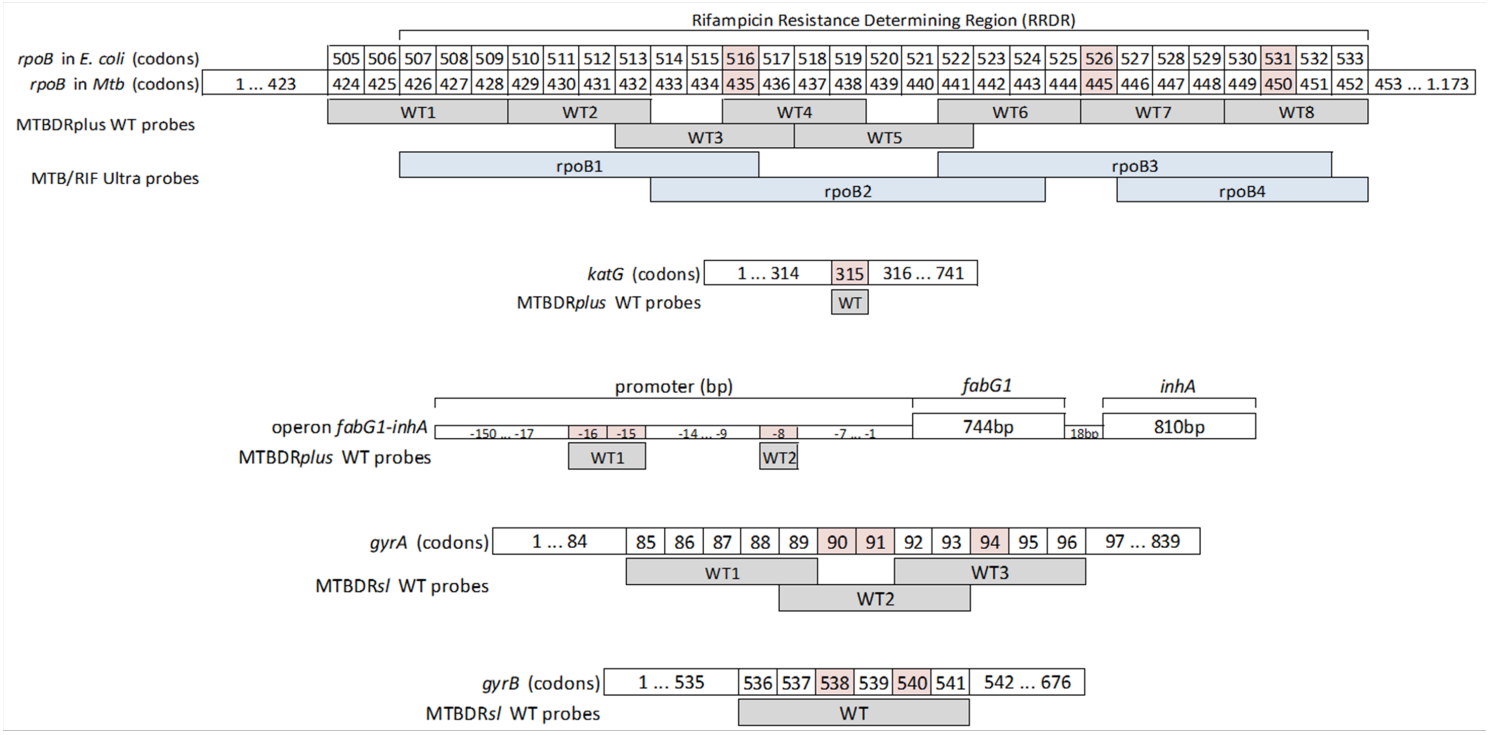


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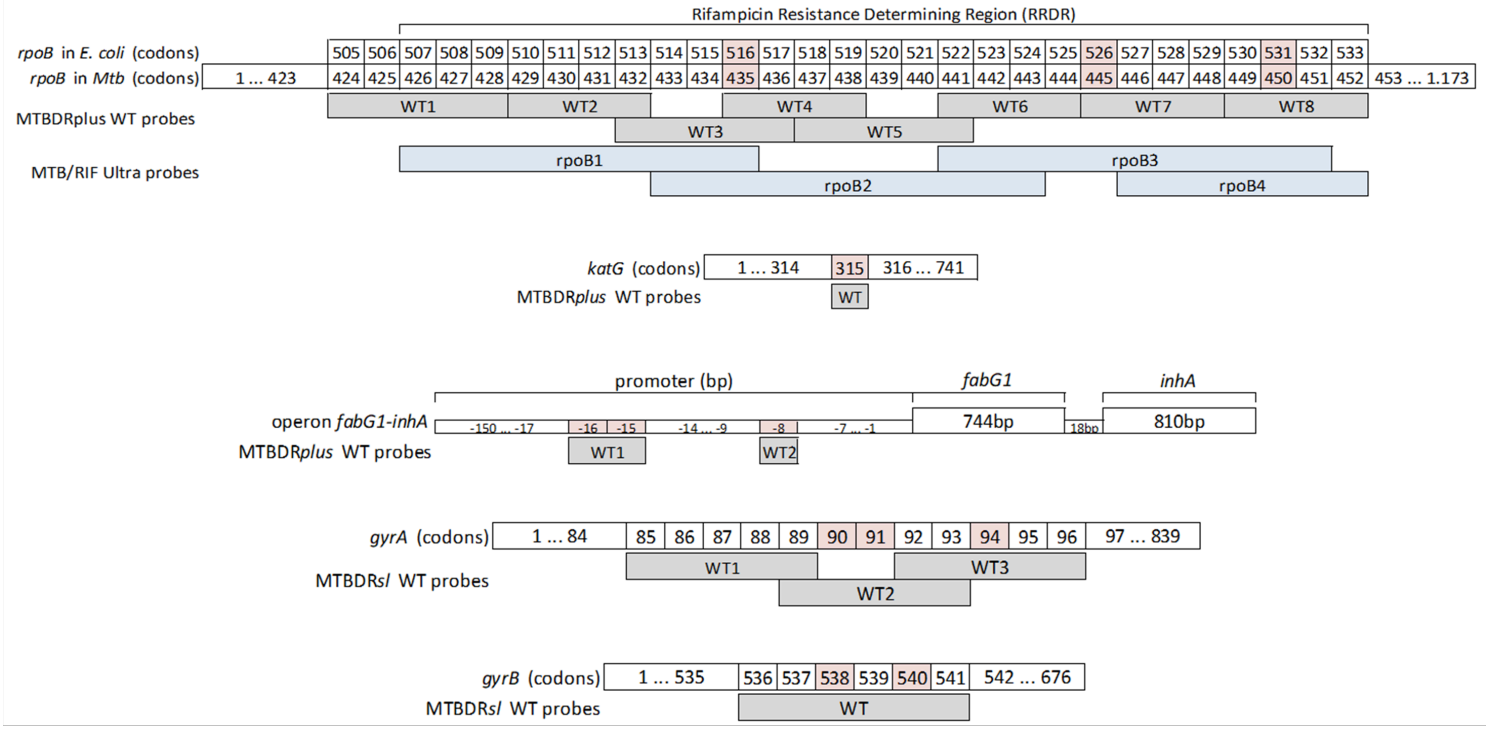


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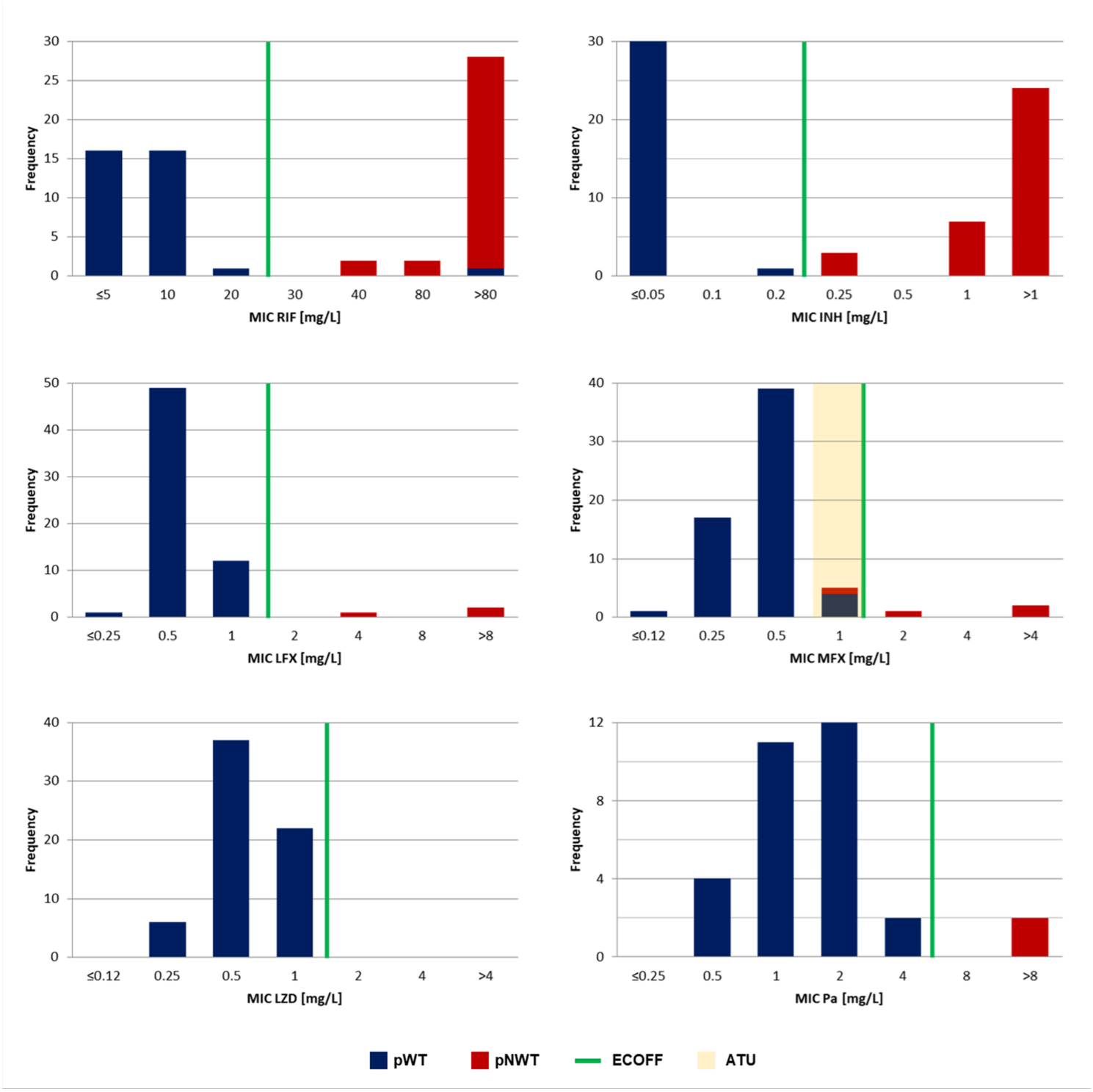


Figure 3

MIC results in Ogawa-Kudoh medium.

MIC: minimum inhibitory concentration; RIF: rifampicin; INH: isoniazid; LFX: levofloxacin; MFX: moxifloxacin; LZD: linezolid; Pa: pretomanid; pWT: phenotypically wild type; pNWT: phenotypically non-wild type; ECOFF: epidemiological cut-off value; ATU: area of technical uncertainty; Blue bars: pWT strains based on EUCAST reference method results; Red bars: pNWT strains based on EUCAST reference method results; Green line: ECOFF based on MIC in Ogawa-Kudoh results; Yellow area: ATU based on overlapping of pWT and pNWT results.

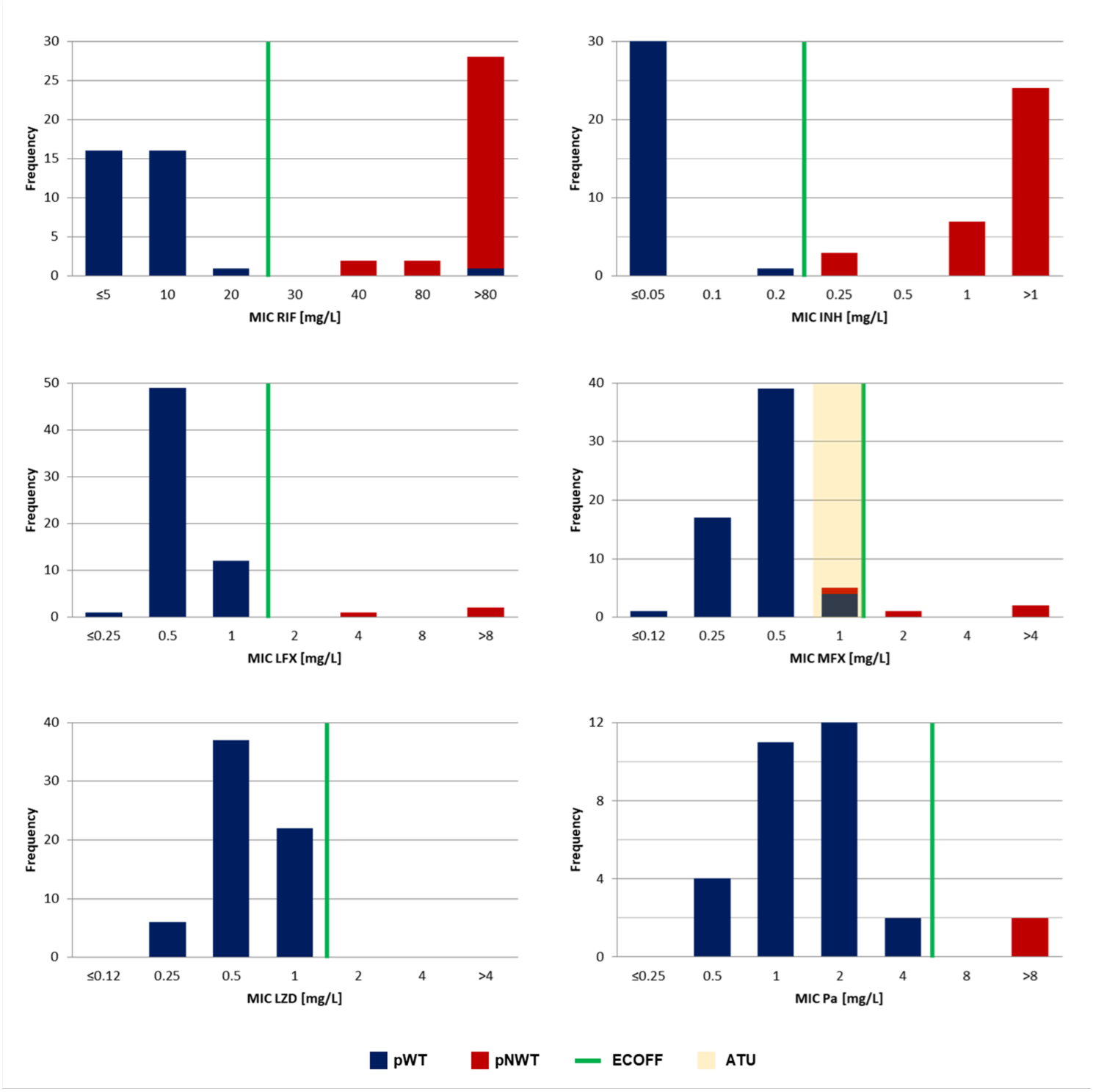


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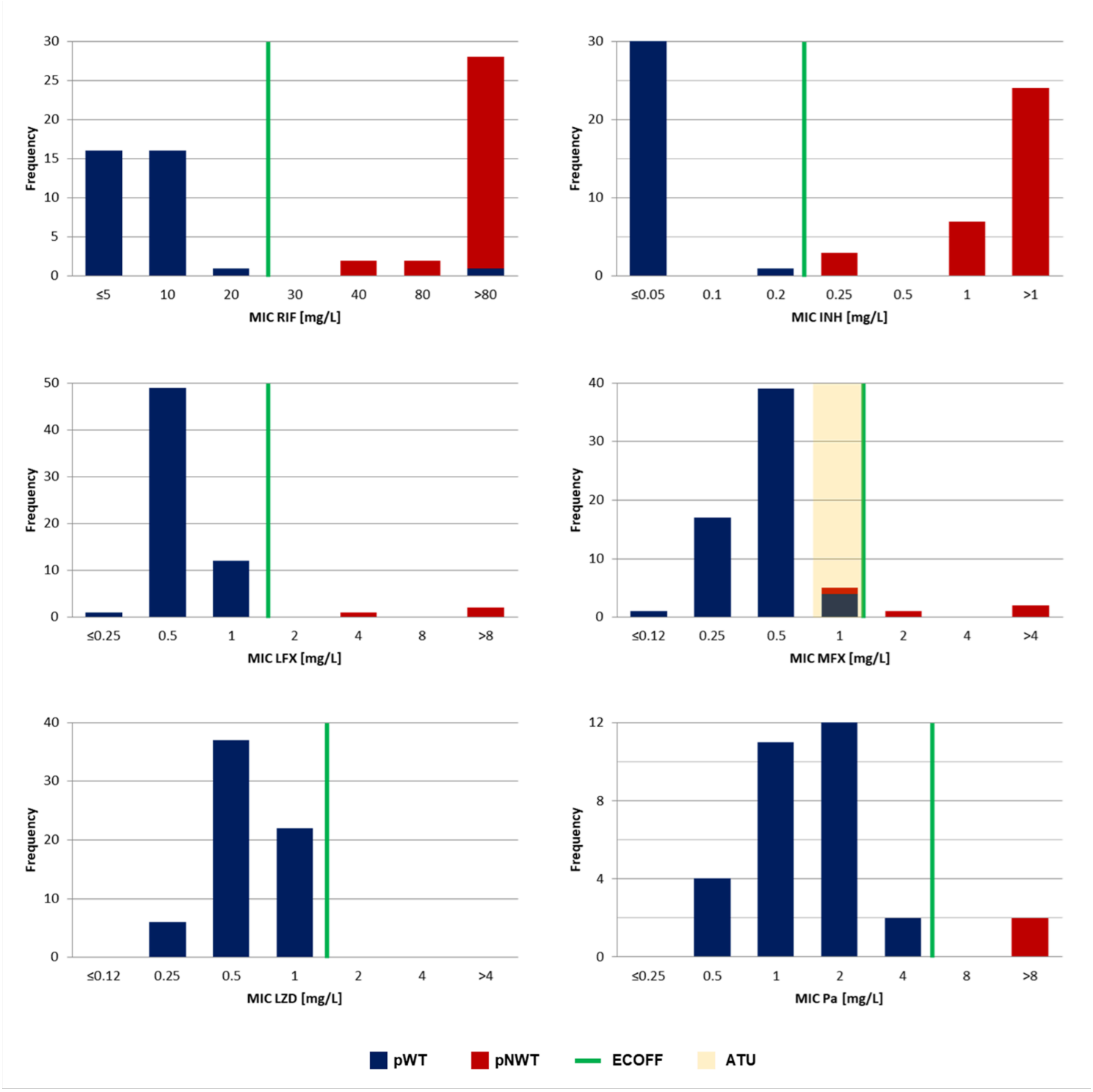


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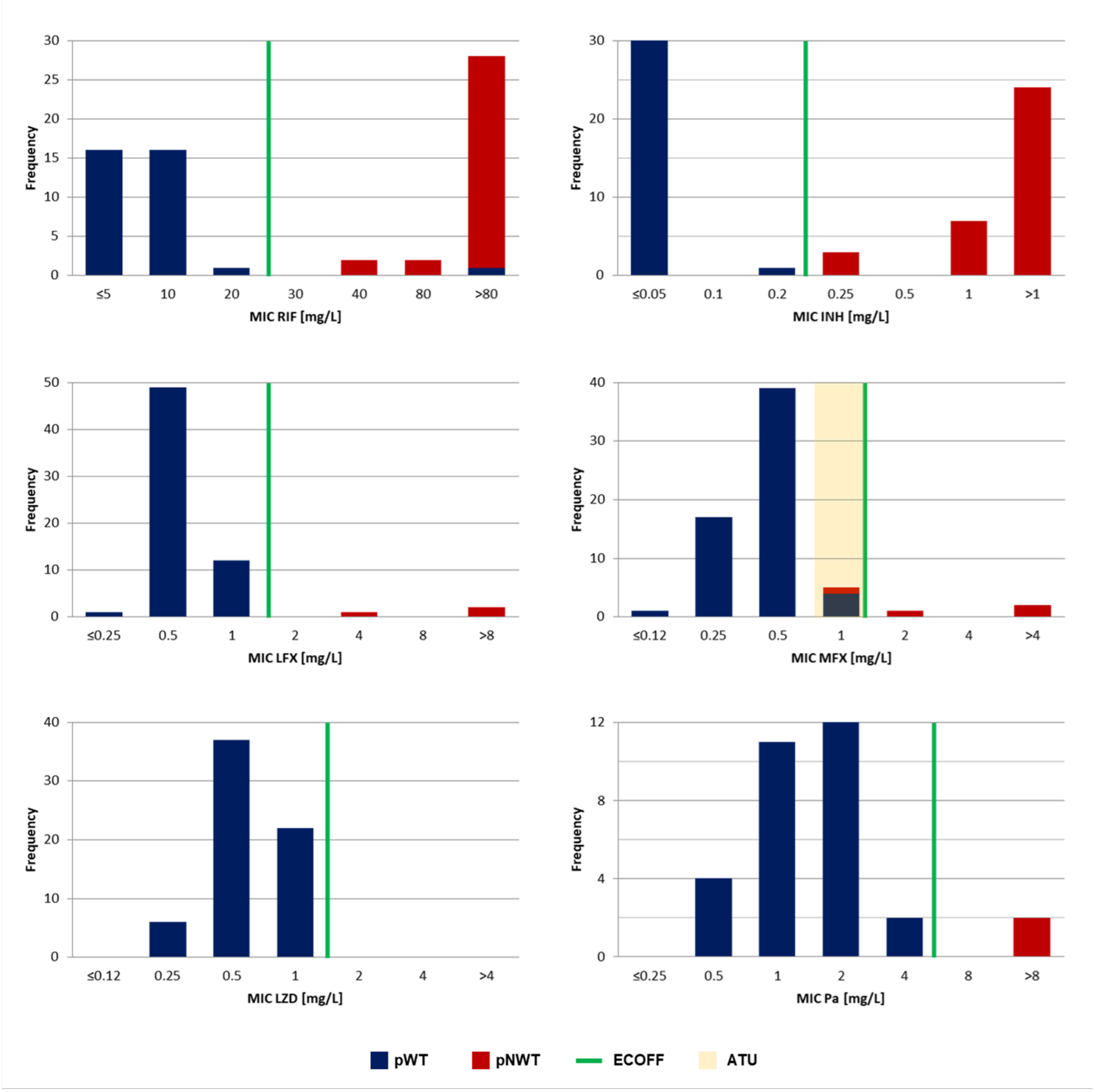


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