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Protocol for the selection of *Mycobacterium tuberculosis* spontaneous resistant mutants to D-cycloserine

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A R T I C L E   I N F O

Method name:
DCS'MCIB

Keywords:
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A B S T R A C T

Mycobacterium tuberculosis (MTB) is known for its adaptive capability in developing resistance to antibiotics, through the selection of spontaneous mutations that arise during treatment. Generating spontaneous antibiotic-resistant mutants *in vitro* is challenging but necessary for studying this phenomenon. A protocol was designed and tested to select stable, MTB spontaneous, d-cycloserine (DCS) resistant mutants. Twenty-four colonies resistant to DCS were selected, demonstrating an increase between 1 and 4 times the Minimum Inhibitory Concentration (MIC) set for *Mycobacterium tuberculosis* H37Rv ATCC 27294 reference strain.

Specifications table

Subject area:	Immunology and Microbiology
More specific subject area:	Microbiology
Name of your protocol:	Selection of *Mycobacterium tuberculosis* Spontaneous Resistant Mutants to d -Cycloserine
Reagents/tools:	**Strain**

- *Mycobacterium tuberculosis* H37Rv ATCC 27294.

Reagents

- Middlebrook 7H9 broth base (Becton Dickinson and Company, Sparks, MD, USA).
- Middlebrook 7H10 agar base (Becton Dickinson and Company, Sparks, MD, USA).
- 85% Glycerol (EMSURE® Sigma-Aldrich).
- Ultra-Pure Glycerol (EMSURE® Sigma-Aldrich).
- OADC Supplement (oleic acid, albumin, dextrose, catalase) (Becton Dickinson GmbH. Heidelberg/Germany).
- Cycloserine (SIGMA C-6880).

Materials

- Petri dish 2 compartments (NEST®).
- Petri dish 4 compartments (NEST®).
- Pipette Filter tips (Axygen®).
- 4 mm glass beads (Paul Marienfeld GmbH & Co. KG. Am. Germany).
- Falcon® 50 mL Conical Centrifuge Tubes.
- Tissue Culture Plate, 96 well U-Bottom with Low Evaporation Lid. Falcon® REF 353,077.
- Microtubes 1.5 ml (Eppendorf®).
- Spreader Blue Polystyrene l-Shaped Spreader (© 2023 Thermo Fisher Scientific Inc).

Tools

- Laminar Flow Cabinet (Esco class II BSC, Esco Micro Pte. Ltd.).
- Thermoshaker (Unimax 1010, Heidolph Instruments GmbH & Co. KG (Unimax 1010. Heidolph Instruments GmbH & Co. KG. Schwabach. Germany).
- CO2 Incubator (Thermo Scientific).
- Pipettes:
  - ❖ PIPETMAN Multichannel P8×200, 20–200 µL (Gilson® PIPETMAN®).
  - ❖ PIPETMAN Metal Ejector (Gilson® PIPETMAN®).
- Fridge (Imbera).
- 20 °C freezer (Haier Biomedical).
- - 80 °C freezer (Panasonic).
- Vortex shaker type mx-s (DLab. Lab Brands S.A.S.© 2021 Lbpro.co).
- Tuttnauver autoclave.

Experimental design:

1. Using the EUCAST method (Fig. 1) [1]. Determine the MIC of H37Rv to DCS.
2. Adjust the inoculum to an optical density 0.55–0.65 McFarland.
3. Conduct an assay using Middlebrook 7H10 agar base (7H10) with DCS concentrations ranging from 0.5X to 7.5X of the MIC obtained in step 1. Include concentrations at 3.75X, 5X, 6.25X.
4. Select individual colonies that grow at the highest antibiotic concentration. Code the selected colonies based on the order and concentration at which they were isolated. For instance, '50–1' (Colony 50–1) signifies the first colony isolated at a concentration of 50 mg/L for DCS.
5. Incubate for 12–16 weeks in Middlebrook 7H9 broth base (7H9) and 7H10 as follows:
   (a) Inoculate an individual colony into 7H9 at a concentration 10 mg/L lower than that of selected colony . For example, Colony 50–1 was inoculated into 7H9 at a concentration of 40 mg/L DCS.
   (b) Incubate with shaking at 140 rpm and 37 °C for 3–4 weeks, denoted as R1, representing the first generation.
   (c) Check the broth every 3–5 days until turbidity appears in R1, use a 4-compartment box containing 7H10 without antibiotics, along with 30, 40, and 50 mg/L DCS.
   (d) Inoculate 100 µL of R1 culture into each compartment. Incubate for 3–4 weeks in a CO_2 atmosphere. This second generation is referred to as R2.
   (e) From the R2 culture, select an independent colony that has grown at the highest concentration. Inoculate it similarly as in **step (a-b)**. This culture is identified as R3, denoting the third generation.
   (f) Repeat **step (c-d)**, using R3, incubating it in the same manner. Select the colony that has grown at the highest concentration to determine the MIC.
6. Subsequently, conduct phenotypic resistance confirmation tests on the colonies using the EUCAST method (Fig. 1) [1]. This step ensures thorough validation of resistance.

Trial registration: N/A
Ethics: N/A
Value of the Protocol:

1. It takes four generations to eliminate colonies that do not exhibit consistent resistance with the initial phenotypic expression.
2. This protocol is valuable for obtaining a complete generation of spontaneous mutants with phenotypic characteristics worthy of evaluation.
3. The generation of spontaneous antibiotic-resistant mutants is a crucial process in the development and validation of phenotypic and molecular susceptibility tests for the early detection of resistance in MTB. This process also provides valuable insights into the genetic basis of resistance, the dynamics of its generation, and potential synergies with other antibiotics. By studying these mutants, researchers can enhance our understanding of antibiotic resistance

Description of protocol

D-cycloserine (DCS) is among the drugs recommended by the World Health Organization (WHO) since 2018 for treating tuberculosis resistant to treatment with first-line drugs [2]. DCS is a drug introduced in 1952, analog of d-ala, its antibacterial mechanism is to inhibit the synthesis of mucopeptides, cell wall constituents (ChEBI & ELIXIR Core Data [3]). Its main advantage is that it has no cross-resistance to other drugs, is widely available and is easily absorbed. Its main disadvantage is neuropsychiatric toxicity, particularly psychosis and seizures, which are seen in 10–50% of patients [4]. These adverse events may depend on the concentration at which the drug is used, so it is imperative to identify doses that can destroy MTB at concentrations lower than those currently associated with toxicity [5].
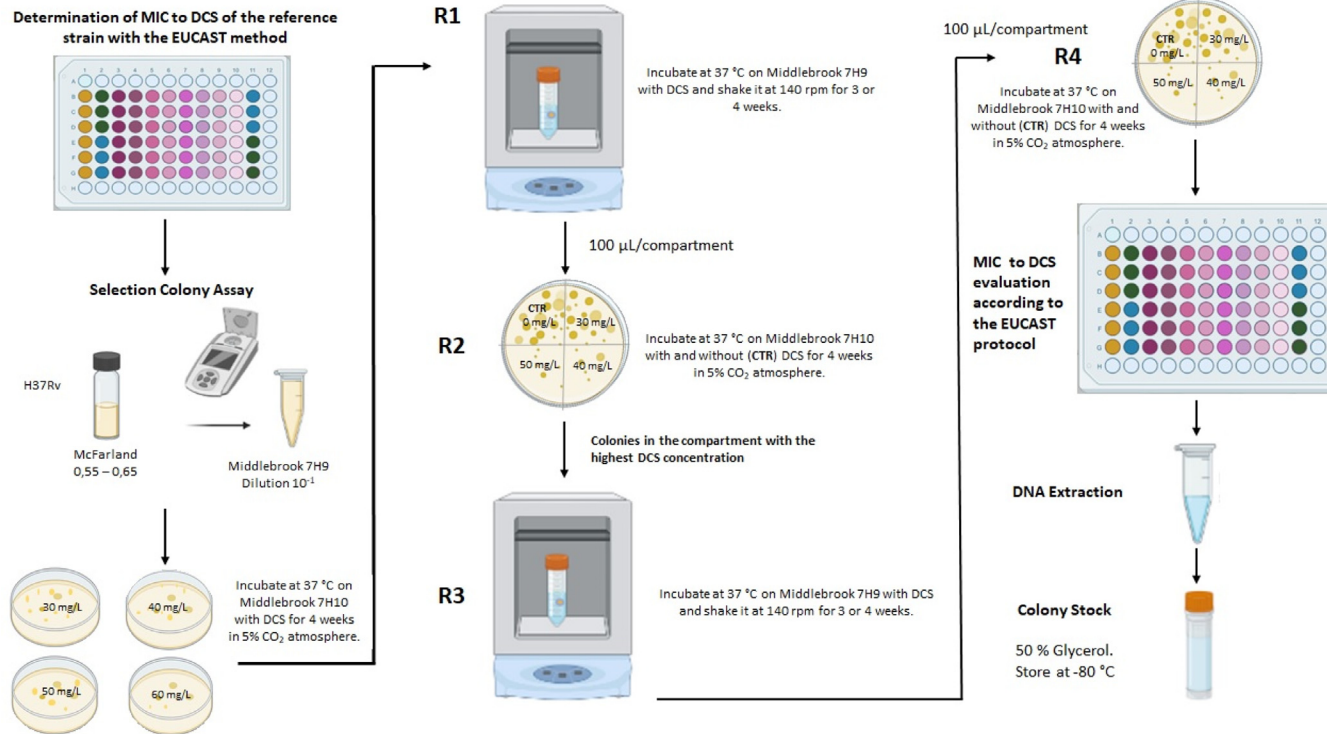
Fig. 1. Methodology for *in vitro* generation and confirmation of resistant mutants. Schematic methodology used to generate and confirm *in vitro* resistant mutants of the *Mycobacterium tuberculosis* (MTB) H37Rv 27294 reference strain.

The selection of spontaneous MTB mutants *in vitro* for DCS enables the acquisition of colonies useful in subsequent phenotypic studies. Given the rarity of resistance to this drug and the absence of an epidemiologic breakpoint (ECOFF), determining whether an isolate is sensitive, is challenging. Therefore, it is necessary to develop phenotypic assays with these mutants in order to improve the studies performed and find the different resistance mechanisms, as Nakatani et al. did in 2017, linking this resistance to mutations in the *alr* gene [6]. Although studies have been carried out with similar protocols testing other anti-tuberculosis drugs [7], this protocol focuses on evaluating and testing the selection of mutants to propose a MIC for DCS and subsequently evaluating these isolates at the molecular level. These improved methods can significantly contribute to the study of resistance to this drug. In addition, the DNA analysis of the mutants could be used to identify the genetic variants present that could explain the phenotypic behavior of the colonies [7,8]. This protocol was developed to select MTB mutants resistant to DCS, proving valuable for investigating the phenotypic aspects of resistance to this antibiotic.

Selection of Spontaneous Antibiotic-Resistant Mutants to d-Cycloserine in *Mycobacterium tuberculosis*

Inoculum preparation

Grow the H37Rv in 5 ml of 7H9 supplemented with oleic acid, albumin, dextrose, and catalase (OADC) and incubate at 37 °C until reaching an optical density of 0.5 on the McFarland scale.

(a) A vial of H37Rv, susceptible to all drugs and stored at −80 °C, should be transferred to −20 °C for 24 h, then to 4 °C for 2 h, and finally to room temperature for 15 min.
(b) Transfer 100 μL of the H37Rv stock from **step (a)** to tubes containing 3 mL of 7H9 supplemented with 0.5% glycerol and 10% of OADC in sterile tubes with 3 glass beads of 4 mm, mixing gently until desegregation occurs.
(c) Incubate by shaking at 140 rpm at 37 °C until visual growth detection (after 2–3 weeks).
   NOTE: Prepare the medium according to the manufacturer's instructions(Becton Dickinson and Company, Sparks, MD, USA).
(d) Confirm purity of the reference strain by using AFB smears.

Antibiotic **stock preparation**

Prepare antibiotic stocks following the recommendations of the EUCAST method.
D-Cycloserine (DCS)

(a) Prepare a 50.000 mg/L DCS (SIGMA C-6880) stock solution using sterile distilled water as a solvent.
(b) Dispense small volumes of the stock solution into sterile cryovials and store at −80 °C until use, for 12 months, or until the expiration date of the original powdered drug.
   NOTE: When removed from the freezer, it must reach room temperature before use.
(c) Check sterility by incubating a small aliquot (5 ml) at 37 °C for 48 h.
(d) Register the date of preparation and antibiotic concentration.

Minimum inhibitory concentration (MIC)

(a) Establish the MIC of DCS for H37Rv to determine the concentrations used in the assays. Initial testing is performed at 0.5X the established MIC, increasing according to the behavior of the isolated colonies.
(b) Follow EUCAST guidelines to determine MIC values.

   • The MIC for the DCS in H37Rv according to the EUCAST method is set at 8 mg/L.

Media stock preparation

Broth media

(a) Prepare and autoclave 7H9 according to the manufacturer's instructions (Becton Dickinson and Company, Sparks, MD, USA).
(b) After autoclaving, allow it to cool to 50 °C.
(c) Add 10% OADC pre-warmed to room temperature (RT; 18–22 °C).
   NOTE: For example, to obtain the different concentrations, prepare 250 mL of base medium and after adding the OADC, divide it into 5 vessels (50 mL) and add the DCS stock solution to each vessel according to the desired concentration, 3.75X (30 mg/L), 5X (40 mg/L), 6.25X (50 mg/L), and 7.5X (60 mg/L). To prepare the 50 mg/L concentration, add 50 μL of DCS stock (50.000 mg/L) to 50 ml of preparation.
(d) Store at 4–8 °C until used.
(e) Register the date of preparation and antibiotic concentration.
(f) Check sterility by incubating a small aliquot (5 ml) at 37 °C for 48 h.
(g) Allow it to reach room temperature before use.

NOTE: In this work, 50 ml of the concentration of interest was prepared as a stock. A 5 ml aliquot was used for several assays. Perform this procedure just prior to testing; ideally, the medium with antibiotics should not be stored for more than 4 weeks.

Solid media

(a) Prepare and autoclave 7H10 according to the manufacturer's instructions (Becton Dickinson and Company, Sparks, MD, USA).
(b) After autoclaving, allow it to cool to 50 °C.
(c) Add 10% OADC pre-warmed to room temperature (RT; 18–22 °C).
(d) Add solutions according to the required concentration on the media: 3.75X (30 mg/L), 5X (40 mg/L), 6.25X (50 mg/L), and 7.5X (60 mg/L). For example, to prepare the 50 mg/L concentration, add 50 µL of DCS stock (50.000 mg/L) to 50 ml of preparation.
NOTE: Remember, the antibiotic stock should not be refrigerated for more than two weeks to prevent degradation. Let the 7H10 solidify and store at 4–8 °C until used. Register the date of preparation and antibiotic concentration on each plate. Check sterility by incubating one plate at 37 °C for 48 h. Allow it to reach room temperature before use.

Selection of spontaneous mutants

a. Transfer 100 µL of the H37Rv initial culture grown in **step 1** to sterile tubes containing 3 mL of supplemented 7H9 OADC and 3 glass beads of 4 mm.
b. Incubate at 37 °C with agitation at 140 rpm for 2 weeks to reach exponential growth phase (optical density of approximately ~0.5 at 600 nm).
c. Adjust the inoculum to an optical density of approximately ~ 0.55–0.65 McFarland (PhoenixSpec Nephelometer, Becton Dickinson and Company, Sparks, MD, USA) ($\sim 3 \times 10^8$ CFU / ml).
d. Prepare a 10^{-1} bacterial dilution by adding 100 µL of the initial bacterial inoculum to 900 µL of 7H9.
e. Transfer 100 µL of undiluted bacterial suspension (previously standardized to McFarland scale (**step 1**)) and 10^{-1} diluted bacterial suspension to 7H10 with different DCS concentrations. Prepare individual cultures for the 10^{-1} diluted bacterial solution and the undiluted bacterial solution, inoculating 7H10 OADC agar with the different concentrations of antibiotic.
f. For each selected antibiotic concentration, 50 µL of the inoculum (obtained in **step b**, the purpose of this is to obtain separate colonies for selection) is inoculated directly onto each side of a two-compartment petri dish to obtain a replicate.
NOTE: One Petri dish for each antibiotic concentration selected for testing.

Incubate at 37 °C with 5% carbon dioxide for 5 weeks or until visual growth detection. Check weekly to select the possible mutant colonies that appear in that period Fig 2.
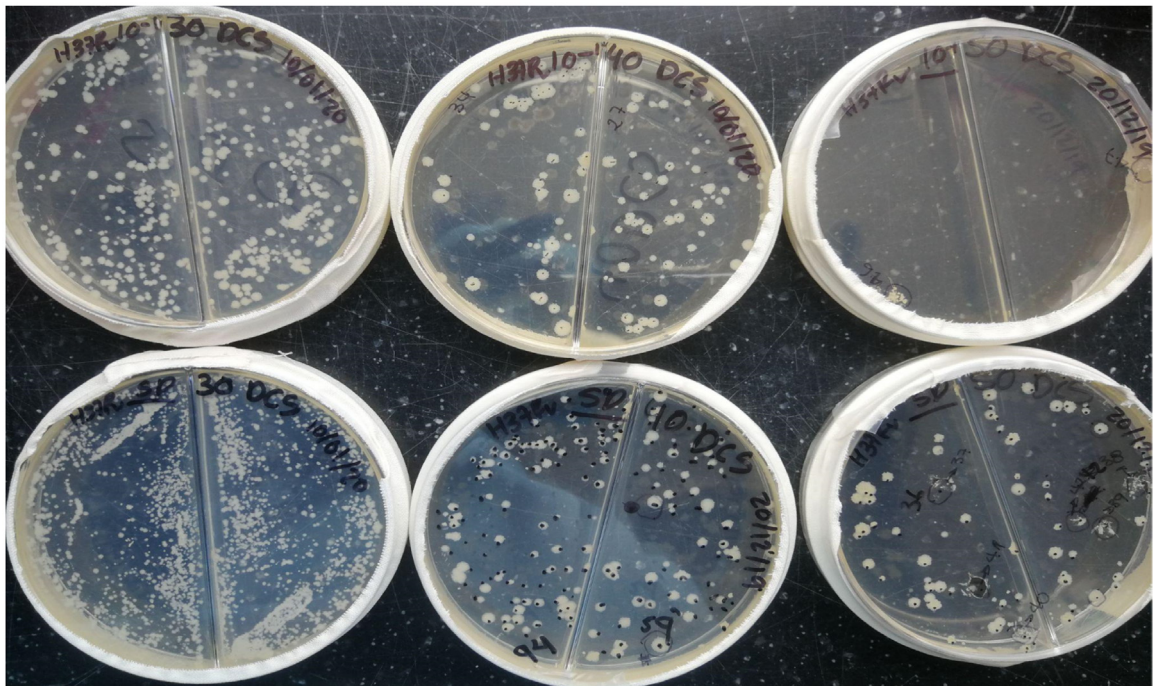

Fig. 2. Selection test for individual colonies. Fig. 2 illustrates the first step of this protocol, the initial assays for selecting *in vitro* mutant single colonies resistant to DCS. H37Rv in undiluted and 10^{-1} diluted cell states, was seeded in duplicate in two-compartment boxes at concentrations of 0, 30, 40, 50, and 60 mg/L DCS. The colonies selected here are the candidates to be evaluated in the subsequent steps of the protocol.

Individual colony selection and subculturing process

- Individual colonies growing at the highest antibiotic concentration (**step f, Selection of Spontaneous Mutants**) are selected through microscopy to verify individuality and are subsequently coded. Each selected colony is assigned a code comprising the DCS concentration at which the colony was obtained and a sequential number (e.g., 50–1 for the first colony at 50 mg/L).

  (a) The coded colonies are then removed using a wooden toothpick and disaggregated by vortexing in a 50 mL Falcon® tube containing 5 mL of 7H9 and 3 sterile glass beads of 4 mm. The medium used for disaggregation contains 1X less antibiotic concentration than the isolation medium (e.g., Colony 50–1 use 40 mg/L concentration in the liquid media), as variations have been observed between solid and liquid media [9]. The resulting culture, designated as **R1 (Subculture 1)**, is incubated for 3 to 4 weeks at 37 °C with shaking at 140 rpm.

  NOTE: Purity of the reference strain is confirmed using acid-fast bacilli (AFB) smears.

  (b) Subculture **R2 (Subculture 2)** is prepared by adding 100 μL of **R1** to a 4-quadrant plate containing 7H10 at three desired concentrations based on the obtained results. One quadrant is left without antibiotics as a growth control. Incubation is carried out for 4 to 5 weeks at 37 °C in an aerobic atmosphere enriched with 5% carbon dioxide, with weekly checks.
    - Selected concentrations of DCS include 0 (no antibiotic used), 30 mg/L, 40 mg/L, and 50 mg/L.

  (c) If an individual colony exhibits growth at a concentration greater than or equal to the isolated concentration in the initial assay e.g., If colony 30–2 grows in **R2** at least in the 30 mg/L quadrant, is candidate to the next step: A single colony is picked from the solid medium, and the same procedure as in **Step (a)** is followed. This resulting culture is designated as **R3 (Subculture 3)**.

  (d) Subculture **R4 (Subculture 4)** is prepared by adding 100 μL of **R3**, following **Step (b)**. If phenotypic resistance persists, the colony becomes a candidate for MIC determination.
  NOTE: Carefully seal and store the assay in which the "resistant colony" was obtained, and keep the remaining colony as a backup Fig. 3.

  (e) The determination of the MIC for DCS should follow the EUCAST protocol (Reference protocol for MIC determination of anti-tuberculous agents against isolates of the *Mycobacterium tuberculosis* complex in Middlebrook 7H9 broth, Version 6.1, 4th of July 2019). This determination should be made in colonies where growth persists at a concentration greater than or equal to that observed at the initial stage Figs. 4 and 5. For this purpose the 7H10 drug-free quadrant should be utilized, the purpose is to obtain separate colonies in an antibiotic-free medium to obtain a susceptibility profile that is not influenced by external factors such as antibiotics in the medium.

  (f) It is recommended to prepare stocks of each colony in 50% ultrapure glycerol and store them at −80 °C.

Protocol Validation

A total of 174 colonies were obtained in a series of 20 assays. Of these, 49 were preselected as colonies potentially resistant to DCS; however, if a decrease in MIC was observed, they were excluded from the study. Further analysis revealed that 24 colonies maintained consistent resistance levels over time at DCS concentrations of 30 mg/L (2 colonies), 40 mg/L (14 colonies), and 50 mg/L (8 colonies). Fig. 6 shows the results of the MIC tests in the 24 colonies after being performed in triplicate using the EUCAST method.

These 24 colonies were stored at −80 °C in the MicroCIB Biological Collection for future genomic analysis.
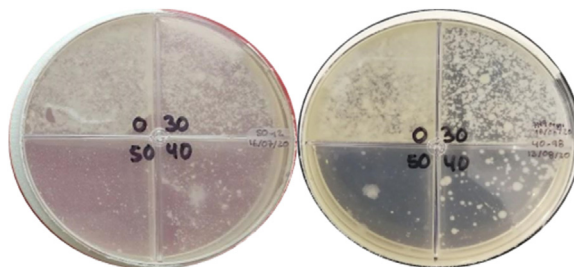

Fig. 3. Colonies 50–12 and 40–98 in Subculture R4. The numbers in the center of the petri dish indicate the concentrations in each quadrant (0, 30, 40, and 50 mg/L of DCS, respectively). There is clear evidence of growth for at least one colony up to the concentration of 50 mg/L in both colonies.
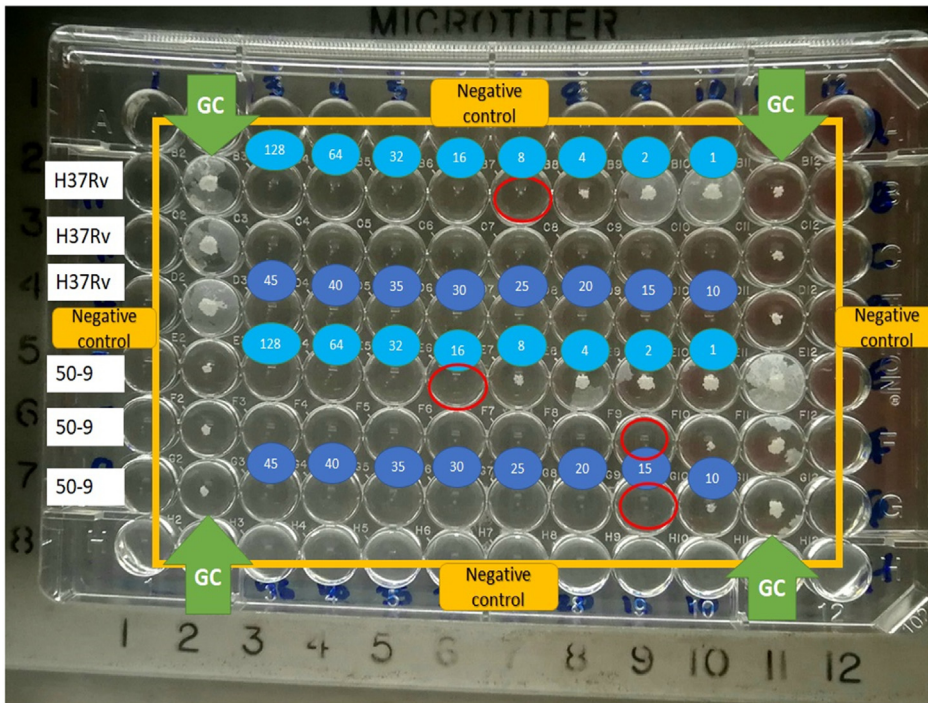
Fig. 4. Comparison of Minimum Inhibitory Concentration (MIC) Results for Reference Strain H37Rv and Colony 50–9. In Fig. 4, the MIC results are juxtaposed with the DCS outcome for both the H37Rv and colony 50–9 after three weeks of incubation. The yellow box represents the negative control, while the green arrows indicate growth controls (GC), EUCAST method [1]. Blue circles denote the evaluated DCS concentrations in the respective wells, and red circles depict the MIC results for these two colonies. For reference strain H37Rv, the MIC is 8 mg/L, while for colony 50–9, it is 16 mg/L.
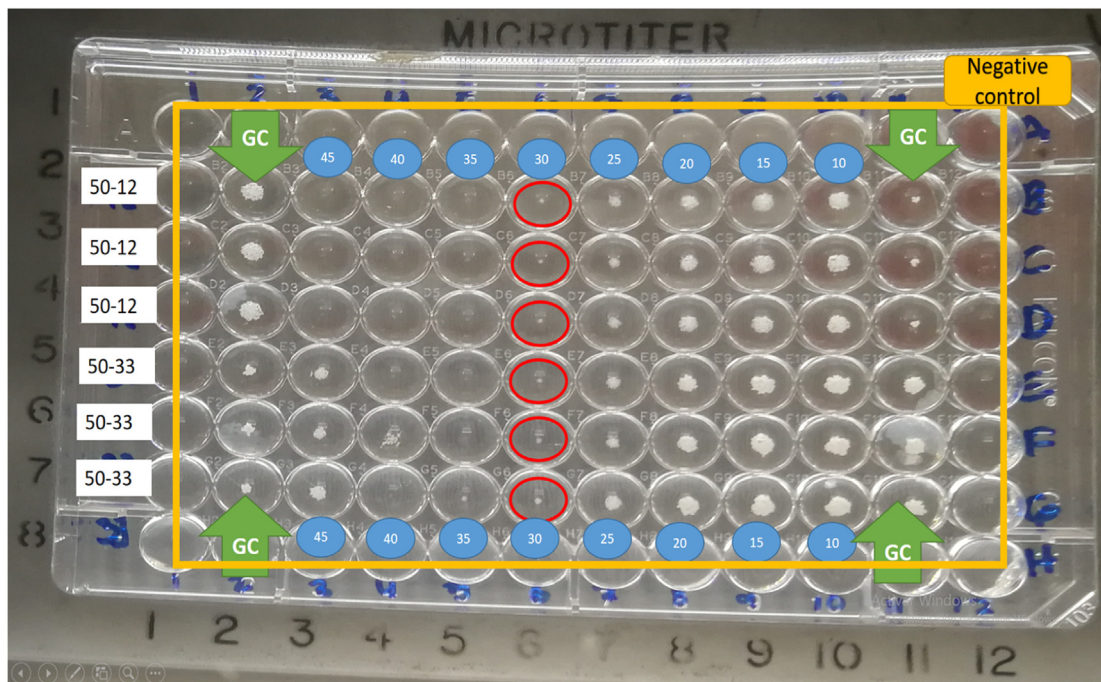

Fig. 5. Minimum Inhibitory Concentration (MIC) to d-Cycloserine (DCS) Results for Colonies 50–12 and 50–33. In Fig. 5, MIC results are presented for colonies 50–12 and 50–33, after three weeks of incubation. The yellow box represents the negative control, while the green arrows indicate growth controls (GC), EUCAST method [1]. Blue circles indicate the evaluated DCS concentrations in the respective wells, and red circles depict the MIC results for both colonies, which was determined to be 30 mg/L for both.
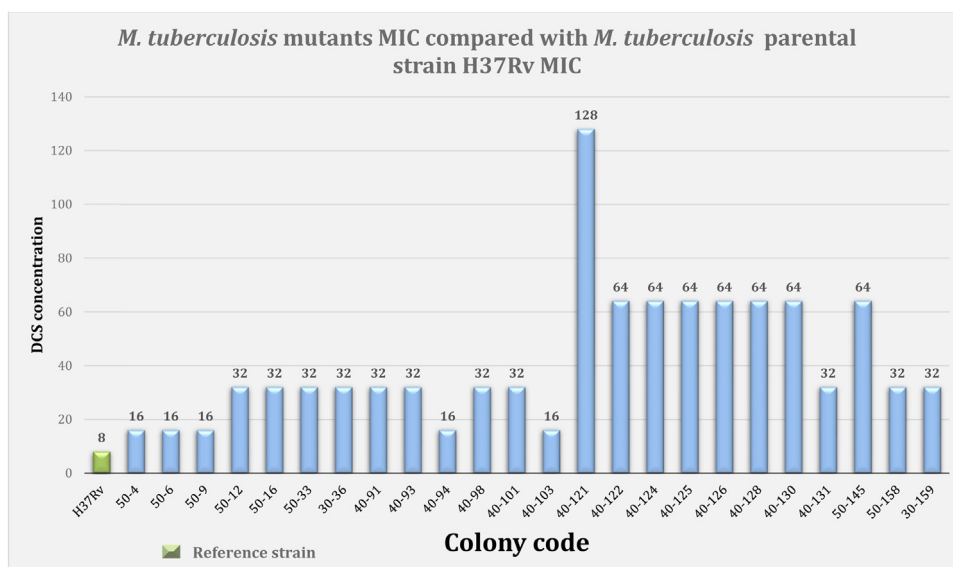
Fig. 6. Minimum Inhibitory Concentration (MIC) results in relation to the H37Rv strain. The figure visually displays the MIC values for each selected colony, along with the reference strain used in the study. The MIC values have shown an increase ranging from 2 to 16 times when compared to the H37Rv strain.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

Alejandra Osorio-González: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing – original draft, Writing – review & editing. **Nataly Álvarez:** Conceptualization, Methodology, Validation, Formal analysis, Writing – review & editing, Project administration, Funding acquisition. **Teresa Realpe:** Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing – review & editing, Funding acquisition. **Jaime Robledo:** Conceptualization, Formal analysis, Writing – review & editing.

Data availability

No data was used for the research described in the article.

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