**Mycobacterium tuberculosis** Strains H37ra and H37rv have Equivalent Minimum Inhibitory Concentrations to Most Antituberculosis Drugs

M. Tobias Heinrichs123, Robert Justin May1, Fabian Heider1, Tobias Reimers123, Sherwin Kenneth B. Sy1, Charles Arthur Peloquin234, Hartmut Derendorf1

Departments of 1Pharmaceutics and 2Pharmacotherapy and Translational Research, University of Florida, 3Emerging Pathogens Institute, University of Florida, 4Infectious Disease Pharmacokinetics Laboratory, University of Florida, Gainesville, Florida, USA

**Abstract**

**Background:** *Mycobacterium tuberculosis* (*Mtb*) strains H37Ra and H37Rv are commonly used to study new and re-evaluate old antituberculous agents with respect to their pharmacodynamic effects *in vitro*. The differences in membrane proteins and, in particular, differences in carrier proteins between *Mtb* H37Ra and *Mtb* H37Rv may have an impact on antibiotic potency. The question of whether H37Ra can be used as a reliable surrogate for H37Rv and clinical strains has not been addressed sufficiently. The purpose of this study is to provide a full comparison of susceptibility data of the most common antituberculosis (TB) agents against both *Mtb* strains. **Methods:** In addition to a literature review, *in vitro* checkerboard susceptibility study was conducted comparing the *in vitro* minimum inhibitory concentration (MIC) of 16 common antituberculous drugs against H37Ra and H37Rv. Heifets–Sanchez TB agar drug susceptibility plates were utilized. **Results:** Half of the antibiotics demonstrated similar growth inhibition against both strains, while slightly differing MIC values were found for 7 of 16 drugs. With the exception of rifampicin, no marked difference in MIC against H37Ra and H37Rv was observed. **Conclusion:** While neither the attenuated (H37Ra) nor the virulent strain (H37Rv) is a clinical strain, both strains predicted MICs of clinical isolates equally well, when comparing the current *in vitro* results to clinical susceptibility data in the literature. H37Ra comes with the benefits of lower experimental costs and less administrative barriers including the requirement of a biosafety Level III environment.

**Keywords:** Attenuated strain H37Ra, minimum inhibitory concentration, *Mycobacterium tuberculosis*, pharmacodynamics, virulent strain H37Rv

**INTRODUCTION**

Tuberculosis is a disease caused by *Mycobacterium tuberculosis* (*Mtb*) and still a leading cause of death in countries with low gross domestic product per capita.1 There are two widely used *Mtb* laboratory reference strains, of which both were derived from the *Mtb* H37 parent strain. H37 was isolated in 1905 from the sputum of a tuberculosis patient. In 1935, William Steenkens managed to obtain two different strains based on morphology and virulence by performing a dissociation study on glycerol–egg media of different pH. The study resulted in the emergence of H37Rv (v for virulent) and H37Ra (a for avirulent).2,3 However, it should be noted that *Mtb* H37Ra is regarded as an attenuated strain rather than being completely avirulent because considerable bacterial growth has been observed in macrophages *in vitro*.4 There are several more differences between the two strains; for example, *Mtb* H37Rv has a smooth-colony morphology while *Mtb* H37Ra is rough.5 The attenuated strain also shows a decreased survival rate inside macrophages and under anoxic conditions.6,7 Zheng et al. conducted a full comparative genomic analysis and found 272 genetic variations (insertions, deletions, and single nucleotide variations) between the two strains.8 A recent study from Jena et al. identified 172 proteins with mutations in *Mtb* H37Ra relative to *Mtb* H37Rv; 89 integral membrane proteins and 74 cytoplasmic proteins have amino acid variations.9

**Address for correspondence:** Dr. Marc Tobias Heinrichs, 1345 Center Drive, PO Box 100494, Gainesville, Florida 32610, USA. E-mail: t.heinrichs@ufl.edu

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We conducted a large-scale literature research to examine the range of minimum inhibitory concentrations (MICs) for the four most common antibiotics (rifampicin [RIF], isoniazid [INH], ethambutol [EMB], and pyrazinamide [PZA]) against these two *Mtb* strains. Table 1 showed the results of the search which included an explanation of the method used. Our literature search indicates that there are significantly less experiments in which MIC values for *Mtb* H37Ra were determined than those for *Mtb* H37Rv. Over the years, researchers have developed numerous methods to determine reliable and reproducible MIC values. The most commonly used method appears to be the radiometric method (BACTEC).

The MIC values in Table 1 were not collected from a consistent and standardized experimental procedure as these values vary depending on the chosen method. EMB MIC results are inconsistent ranging from 0.06 to 4 μg/ml against *Mtb* H37Rv and from 0.62 to 3.05 μg/ml against *Mtb* H37Ra. We found that EMB susceptibility evaluation procedures were also quite variable between *Mtb* strains compared to other anti-TB drugs. The first-line anti-TB drug PZA shows strong pH dependency in its potency which resulted in incompatibility between experiments conducted with different pH values of the culture media.[23] Consequently, there are not many reported PZA MIC values against *Mtb* strains compared to EMB. The analysis shows more consistencies for INH and RIF across studies. The BACTEC results for INH against *Mtb* H37Rv from different papers are virtually identical, whereas the MIC results using other susceptibility testing methods against *Mtb* H37Ra strain were not markedly different. The reported RIF MIC values using various methodologies do not exhibit large variability.

Differences between *Mtb* H37Rv and *Mtb* H37Ra are due to the genetic and proteomic differences.[8,9] Differences in membrane proteins and, in particular, differences in carrier proteins may have an impact on the influx and efflux of antibiotics. Even small mutations of target proteins for the antibiotics are also highly likely to have a large impact on the MIC value. In context of this literature review, the reader should refer to the analysis of six *Mtb* H37Rv strains from different laboratories surveyed by Ioerger et al.[5] They demonstrated that genetic differences among those strains evolved through an “in vitro evolution.”[5] Hence, it should be noted that there might be differences even between the reference strain H37Rv from different laboratories depending on how often the isolate has been re-cultured in the laboratory.

Our literature analysis showed a lack of reliable data, especially for *Mtb* H37Ra, and a large discrepancy between MIC values reported in the literature even though similar methods have been used. In addition, the question of whether *Mtb* H37Ra can be used as a reliable surrogate for *Mtb* H37Rv could not be answered sufficiently. Consequently, we generated susceptibility data (MIC values) of antituberculosis (TB) drugs against both *Mtb* strains. The current study examined 16 different anti-TB agents. By comparing the MICs of the most important antibiotics against both strains under the same
the objective of this study is to determine whether the less virulent strain is comparable to the virulent strain in terms of their response to various antimicrobial agents. There are important advantages of working with the attenuated *Mtb* H37Ra strain. Foremost, biosafety Level II is sufficient when working with the attenuated strain, which makes the experiments more cost efficient to run. The information is particularly relevant for high TB burden countries (e.g., sub-Saharan African countries) such that these experiments can also be conducted by organizations in countries that do not have access to biosafety Level III facilities. It should be noted that there are also major genomic differences which may also manifest in phenotypic differences between *Mtb* H37Rv and actual clinical isolates.

**Methods**

**Preparation of drug susceptibility plates**

Drug susceptibility plates were prepared as described by Heifets and Sanchez24,25 in the following doubling concentrations (μg/ml): bedaquiline 0.002–0.5, capreomycin 0.5–8, clofazimine 0.064–0.25, cycloserine 6.25–400, EMB 0.5–8, ethionamide 0.25–4, INH 0.125–0.5, kanamycin 1–16, levofloxacin 0.5–2, linezolid 0.125–2, moxifloxacin 0.25–1, p-aminosalicylic acid 0.125–2, PZA 18.75–75, rifabutin 0.004–0.064, RIF 0.016–0.5, and streptomycin 0.5–8. Concentration ranges were chosen based on reported MIC values against the two *Mtb* strains, evaluated on solid agar medium. Capreomycin, clofazimine, cycloserine, EMB, ethionamide, INH, kanamycin, levofloxacin, p-aminosalicylic acid, PZA, and RIF were purchased from Sigma-Aldrich, St. Louis, MO, US. Bedaquiline was from Advanced ChemBlocks Inc., Burlingame, CA, US; moxifloxacin was from Thermo Fisher Scientific, Waltham, MA, US, and linezolid and rifabutin were from Sequoia Research Products Ltd, Pangbourne, United Kingdom. Culture medium (Middlebrook 7H11 [Sigma-Aldrich] with monopotassium phosphate [Thermo Fisher Scientific], glycerol [Sigma-Aldrich], and bovine calf serum [Sigma-Aldrich] supplement) was identical for both *Mtb* strains and prepared at the same time with the same batch of medium. 7H11 agar base, monopotassium phosphate, and glycerol were dissolved in distilled water by stirring with a magnetic bar on a magnetic stir plate. After autoclaving at 121°C for 15 min, the pH was measured using a pH meter. Agar was cooled down to 54°C in a water bath, and sterile bovine calf serum was added to a final concentration of 10% v/v. The 100 mm × 15 mm Petri dishes (Parter Medical Products Inc., Carson, CA, USA) were divided into 4 separate quadrants [Figure 1]. Cooled medium was poured into quadrant 1 of each plate as a nondrug control, approximately 5 mL/quadrant. For quadrants 2, 3, and 4, sterile-filtered drug solutions diluted from a stock were spiked into liquid TB agar and stirred on a magnetic stirring hot plate. The solutions were then poured into quadrants. Stock solutions were prepared for bedaquiline in acetonitrile, clofazimine and rifabutin in dimethyl sulfoxide, ethionamide in ethylene glycol and H2O, linezolid in ethanol, moxifloxacin and RIF in methanol, and the remaining drugs in deionized water.

**Bacterial culture**

Cultures of *Mtb* were grown from a frozen stock of ATCC 25618 and 25177 strains in a mycobacteria growth indicator tube (MGIT) prepared per package insert and incubated in a BD MGIT machine at 37°C until the machine called it positive plus 2 days, which is equivalent to ~10^6 CFU/mL (confirmed by plating studies). Both strains were grown in Middlebrook 7H9 (Sigma-Aldrich) with 10% Oleic Albumin Dextrose Catalase (Sigma-Aldrich) supplement and 0.05% w/v Tween 80 (Sigma-Aldrich) and were at a similar stage of growth when inoculated onto the plates.

**Inoculation of drug susceptibility plates**

Tubes containing bacterial cultures were vortexed for 20 s and diluted to final concentrations of 10^3 CFU/mL and 10^4 CFU/mL (inoculum concentrations were confirmed through CFU count on agar plates). 0.1 mL of these concentrations was inoculated on each quadrant of the previously prepared agar plates. The plates were then sealed in CO2 permeable plastic bags with a heat sealer. The sealed plates were inoculated at 37°C and monitored weekly for adequate growth of the control quadrant.

**Minimum inhibitory concentration determination**

After 25 days of incubation, plates were removed from the incubator and colonies were counted to compare the control versus the quadrants containing anti-TB agents. MIC was defined as the lowest concentration that resulted in no visible bacterial growth. Each drug concentration was tested in triplicate. The modal MIC value is reported in this study.
RESULTS

Growth inhibition of two Mycobacterium tuberculosis strains in the presence of antituberculosis agents

Eight antibiotic agents demonstrated similar growth inhibition for both the attenuated (H37Ra) and the virulent strain (H37Rv). Among these, agents are capreomycin (8 µg/mL), EMB (8 µg/mL), INH (0.5 µg/mL), kanamycin (4 µg/mL), linezolid (0.5 µg/mL), p-aminosalicylic acid (2 µg/mL), and streptomycin (4 µg/mL). Interestingly, even PZA, being one of the first-line drugs against Mtb infections, showed identical MIC values against both Mtb strains (75 µg/mL).

We also found slightly differing MIC values for several drugs. One would expect that the MIC for bedaquiline, being one of the newer antituberculous agents, should not differ much against both H37Ra and H37Rv. Interestingly, we observed a MIC of 0.064 µg/mL against the attenuated strain and a roughly 2-fold higher value for the virulent strain (0.125 µg/mL). Furthermore, the MIC of clofazimine against H37Rv was higher than the MIC against H37Ra (0.25 µg/mL). Similar observations were made for ethionamide and rifabutin. Furthermore, the MIC of cycloserine was roughly 2-fold higher against the virulent strain than for the attenuated strain (200 µg/mL for H37Ra vs. 400 µg/mL for H37Rv). Finally, we discovered a roughly 8-fold difference in MIC of RIF against H37Rv as compared to H37Ra (0.5 µg/mL vs. 0.064 µg/mL, respectively).

The virulent strain seems to be more susceptible to the two commonly used fluoroquinolones, levofloxacin and moxifloxacin (levofloxacin: 1 µg/mL against H37Ra vs. 0.5 µg/mL against H37Rv and moxifloxacin: 0.5 µg/mL against H37Ra vs. 0.25 µg/mL against H37Rv). All results are shown in Table 2. A bar graph of H37Ra and H37Rv MIC values for all drugs is shown in Figure 2. The pH measured in Heifets–Sanchez TB agar was 6.0–6.1.

H37Ra as a good surrogate for H37Rv

In summary, we observed equivalent MIC values against both strains for half of the agents tested. Both fluoroquinolones showed a 2-fold higher MIC against H37Ra as compared to H37Rv, whereas 4 drugs had 2-fold lower MIC values against H37Ra as compared to H37Rv. However, 2-fold differences are considered within the errors of determination and therefore not considered a significant difference.

With the exception of RIF (being one of the oldest anti-TB drugs), no significant differences were observed between the two strains with respect to drug susceptibility. Thus, overall H37Ra seems to be a good surrogate for H37Rv.

Comparison to clinical susceptibility data – both laboratory strains predict clinical susceptibility equally well

Our results were compared to published susceptibility data of clinical isolates listed in the database from the European Committee on Antimicrobial Susceptibility Testing (EUCAST)(26) and to several other published results for those not being listed in EUCAST. The goal was to identify the strain that better predicts clinical susceptibility as it is the

| Table 2: Minimum inhibitory concentrations of the tested antituberculous drugs in H37Ra, H37Rv, and comparison to literature-reported minimum inhibitory concentration in clinical isolates |
|---|
| **Drug** | **MIC (µg/mL)** | **Comparison** | **MIC (µg/mL)** | **Literature value(s)** | **Clinical isolates** |
| --- | --- | --- | --- | --- | --- |
| Bedaquiline | 0.064 | < | 0.125 | 0.064, 0.002-1(29)* | |
| Capreomycin | 8 | = | 8 | 2, 0.25-64(26)* | |
| Clofazimine | 0.25 | < | >0.25 | 1(26) | |
| Cycloserine | 200 | < | 400 | 8-32(27) | |
| EMB | 8 | = | 8 | 2.5-5(28) | |
| Ethionamide | 1 | < | 0.5 | 0.1-0.2(10,30) | |
| INH | 0.5 | = | 0.5 | 0.1-0.2(10,30) | |
| Kanamycin | 4 | = | 4 | 0.5-512(29)* | |
| Levofloxacin | 1 | > | 0.5 | 0.125-0.5(31) | |
| Linezolid | 0.5 | = | 0.5 | 0.25, 0.064-8(26)* | |
| Moxifloxacin | 0.5 | > | 0.25 | 0.25, 0.032-8(26)* | |
| p-aminosalicylic acid | 2 | = | 2 | 1(26) | |
| PZA | 75 | = | 75 | 32, 16-512(26)* | |
| Rifabutin | 0.016 | < | 0.032 | 0.032, 0.004-16(26)* | |
| RIF | 0.064 | < | 0.5 | 0.125-0.5(30)* | |
| Streptomycin | 4 | = | 4 | 0.5, 0.125-512(26)* | |

*EUCAST data. The mode and observed range of an MIC distribution is shown. MIC distributions were not always normally distributed. INH: Isoniazid, RIF: Rifampicin, PZA: Pyrazinamide, EMB: Ethambutol, EUCAST: European Committee on Antimicrobial Susceptibility Testing, MIC: Minimum inhibitory concentration.

Figure 2: Side-by-side comparison of H37Ra and H37Rv minimum inhibitory concentration values on a logarithmic scale (y-axis) for 16 antituberculosis drugs (x-axis)
susceptibility of clinical isolates that truly matter at the end of the day.

For 8 out of 16 tested drugs (capreomycin, EMB, INH, kanamycin, linezolid, p-aminosalicylic acid, PZA, and streptomycin), the MIC of anti-TB agents against both strains is equally suitable inference of MICs of the same anti-TB drugs against clinical isolates [Table 2]. MIC value determined in H37Ra was a better predictor for 3 out of 16 drugs (bedaquiline, cycloserine, and ethionamide) while MIC against H37Rv was a better predictor for 4 out of 16 drugs (levofloxacin, moxifloxacin, RIF, and rifabutin). No formal comparison was done for clofazimine.

**DISCUSSION**

It should be noted that the MIC we found for p-aminosalicylic acid (PAS) (2 μg/mL) should be taken with caution. It does not match the generally accepted MIC of 1 μg/mL which is often stated in the literature[25,34,35] although 2-fold differences are considered within the errors of determination. Furthermore, PAS does not give 99% inhibition, which contributes to the error of determination, and thus, a wider MIC range may be acceptable. In our literature search, we found that the MIC does not only depend on the medium used but also on the formulation of PAS and the inoculum size.[11] In addition, the method of susceptibility determination plays an important role.[10] In our case, commercially available pure PAS was added to quad plates. Counts were then taken visually with the help of a digital counter pen.

The MIC values observed in this study tend to be higher when compared to previously published work, in particular, when compared to data coming from BACTEC experiments (2–10-fold higher).[11-13] This observation supports the thesis that different experimental systems yield (slightly) differing MIC data. Even with the same methodology being used, there may still be an “interlaboratory variability” with respect to the generated MIC data. There are obvious differences between the experimental system at hand and the BACTEC system that uses liquid medium and a higher pH compared to the acidified pH in Heifets–Sanchez TB agar. However, the primary objective of this study was not to investigate the effect of different experimental systems on the MIC data they produce but to draw a comparison between H37Ra and H37Rv under identical experimental conditions. Heifets–Sanchez TB agar plates were identified as the experimental method of choice as they are easy to prepare, inexpensive, and yield highly reproducible results.

Our study has a number of limitations. There are more aspects to antimicrobial pharmacodynamics, for instance, the kinetics of the drug effects. These could be determined through lengthy time-kill experiments, which was beyond the scope of our study. Further, the Heifets–Sanchez TB agar medium has a lower pH of 6.0–6.1 instead of the usual for agar media, pH 6.8. This may explain, in part, an increased MIC as the antibiotic activity of several antibiotics decreases with decreasing pH (with the exception of PZA).[37-40] The main reason for a lower efficacy (i.e., an increased MIC) at acidified pH may be the dependence of cellular uptake on drug ionization state. Yet, the choice of a lower pH is a reasonable one and even somewhat closer to actual in vitro conditions as clinicians have recently reported a slightly acidified pH in infected lung tissue of TB patients (median: 5.5, range 5.0–7.2).[41]

Although some consider H37Rv the preferred strain for in vitro experiments, our work has shown that H37Ra results predict clinical susceptibility equally well as the H37Rv results. Neither H37Ra nor H37Rv is a clinical strain; however, both strains have been shown to be good in vitro predictors for clinical outcome. The current study addresses one aspect of interchangeability of Ra and Rv strains as inference for susceptibility evaluation of anti-TB agents against clinical isolates from tuberculosis patients. The applicability of the Ra strain as inference can bring significant cost efficiency to laboratories worldwide, given that the use of Ra does not require a biosafety Level III environment.

**CONCLUSION**

The H37Ra strain is equally useful as the H37Rv strain in its response to anti-TB agents and is also inferential of the responses of clinical isolates of tuberculosis to these agents. The H37Ra strain can be used to evaluate potency of anti-TB agents to *Mtb* bacilli.

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**Conflicts of interest**

There are no conflicts of interest.

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