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Determination of Minimum Inhibitory Concentration of Cycloserine in Multidrug Resistant *Mycobacterium tuberculosis* Isolates

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Abstract

This study was performed to determine the minimum inhibitory concentration (MIC) of cycloserine on 48 multidrug resistant tuberculosis (MDR-TB) isolates using the Broth Microdilution Method. No relationship between specific concentrations of cycloserine administrated and the bacterial inoculums used was found. This implied that the clinical isolates were obtained from patients on different treatment regimens. The findings of this study would potentially be important in alleviating the toxic effects of cycloserine in MDR-TB-infected patients while attempting to maintain effective doses.

Keywords: *: Cycloserine, Tuberculosis, Susceptibility, Resistance, Colonies.*

1. Introduction

Mycobacterium tuberculosis (TB) is the etiological agent of tuberculosis in humans. *M. tuberculosis* is a large non-motile rod-shaped bacterium that has a slow generation time (of between 15 and 20 hours), and this contributes to its invasive and aggressive spread in the well-aerated upper lobes of the lungs (Todar, 2011). Patients tend to become drug resistant to tuberculosis drugs or agents when the bacterium mutates (Singh, 2013). In addition, lipids (Mycolic acids), cord factor, and wax-D, that constitute the mycobacterial cell wall, facilitate development of resistance to various drugs (Todar, 2011). Many drug compounds are unable to enter the cell surface of mycobacteria because of this alpha-branched hydrophobic lipid layer, which contributes to its virulence. As a result, this has prompted the design of drugs that can cross this lipid barrier leading to death of the bacterium and allowing it to be destroyed by macrophages (instead of allowing their survival in macrophages as facultative intracellular parasites) (Todar, 2011; Singh, 2013).

Performing and interpreting minimum inhibitory concentrations (MICs) of TB isolates is important to prevent their spread across nations, and particularly in developing countries, where people are living with different stages of this disease and are given different types of treatment regimens. Tuberculosis has the most impact in developing countries because of resource limitations and the low socioeconomic living standards (Singh, 2013).

For this study, the MIC was defined as the lowest concentration at which cycloserine, a second-line, anti-tuberculosis drug, had to be administered in order to hinder the multiplication of tuberculosis isolates. In this study, Multidrug Resistant (MDR)-TB isolates were treated with this drug. Cycloserine ($C_3H_6N_2O_2$ (Sigma, 2008)) is a broad-spectrum antibiotic that is bacteriostatic at the recommended dosage. It is usually purchased either in capsule form or as a whitish/white-yellow powder that can be dissolved completely in water or partially in ethanol (Official Monographs for Part 1). Its usage has become limited particularly because of its hypersensitivity- (WHO PAR Part 4, 2007) and neurologically-related complications (Wolinsky, 1993) that have made the treatment of patients too costly. These costs arise from patients becoming obliged to monthly neuropsychiatric assessments. Some of the neuropsychiatric complications for which patients are treated include: confusion, convulsions, depression, dysarthria, headache, paresis, psychosis, somnolence, tremor, vertigo and the less well-understood human pregnancy/mother breastfeeding cases on treatment (WHO PAR Part 4, 2007). However, in spite of these complications, cycloserine has been used for renal and hepatic treatment in patients (Singh, 2012).

This study was therefore undertaken to determine the MIC of 48 MDR-TB isolates through treatment with five different concentrations of cycloserine (8, 16, 32, 64, >64 µg/mL). Thus, this study is a crucial step in alleviating the toxic effect of cycloserine in MDR-TB-infected patients, while attempting to maintain effective concentrations in the serum of infected patients.

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2. Materials and Methods

2.1. Bacterial Strains

Multidrug resistant tuberculosis (MDR-TB) isolates were frozen in cryovials using 0.5 mL freezing solution that contained 2g protease peptone and 16 mL glycerol. These isolates were retrieved from a reputable hospital in the KwaZulu-Natal area in South Africa. Since this study reports clinical isolates, the experimental names of the isolates have been withheld for confidentiality purposes. The experimental numbers start with the letter 'E' as indicated in the Appendix. $H_{37}R_v$, a susceptible tuberculosis isolate, was used as the control strain in this study because of its capability to utilise drugs efficiently compared to drug-resistant TB isolates. Therefore it was used as a reference when interpreting cycloserine susceptibility results in this study.

Appendix

Table 1. Cycloserine MIC results for clinical and control TB isolates at pH 7.2.

Number	Experiment no.	Day 7	Day 14	Day 21 (MIC)	Day 28	Inoculum (CFU/mL)
1.	E.12.	32	64	64	>64	7×10^6
2.	E.18.	32	32	64	64	4×10^6
3.	E.22.	32	32	64	>64	7×10^6
4.	E.23.	16	32	64	64	6×10^6
A.	$H_{37}R_v$	16	32	32	64	1×10^6
5.	E.25.	8	32	32	32	5×10^6
6.	E.28.	16	32	32	32	8×10^6
7.	E.30.	16	16	32	32	6×10^6
B.	$H_{37}R_v$	8	32	32	32	5×10^6
8.	E.33.	16	16	32	64	7×10^6
9.	E.35.	16	16	16	64	7×10^6
10.	E.39.	16	32	32	64	7×10^6
11.	E.51.	16	16	32	64	1×10^7
12.	E.29.	16	16	32	64	7×10^6
C.	$H_{37}R_v$	8	16	32	32	5×10^6
13.	E.40.	16	32	64	>64	7×10^6
14.	E.15.	>64	>64	>64	64	9×10^6
15.	E.47.	>64	>64	>64	64	8×10^6
D.	$H_{37}R_v$	16	32	64	64	5×10^6
16.	E.31.	32	32	64	64	9×10^6
17.	E.41.	32	32	64	64	7×10^6
18.	E.53.	32	64	>64	>64	7×10^6
E.	$H_{37}R_v$	16	32	64	64	4×10^6
19.	E.36.	16	32	32	64	1×10^7
20.	E.70.	16	32	32	64	1×10^7
21.	E.54.	16	*	64	64	1×10^7
F.	$H_{37}R_v$	8	16	32	32	4×10^6
22.	E.24.	32	32	64	64	7×10^6
23.	E.26.	32	32	64	64	2×10^7
24.	E.14.	16	32	64	64	7×10^6
G.	$H_{37}R_v$	8	16	32	64	8×10^6
25.	E.55.	16	32	32	64	1×10^7
26.	E.60.	16	32	32	64	1×10^7
27.	E.66.	16	32	64	64	2×10^6
28.	E.58.	16	32	64	64	2×10^7
29.	E.10.	16	32	32	64	1×10^7
H.	$H_{37}R_v$	16	16	32	64	1×10^7
30.	E.65.	16	32	64	64	8×10^6
31	E.24.	16	32	64	64	9×10^6
32.	E.71.	64	64	>64	>64	7×10^6
I.	$H_{37}R_v$	16	32	64	64	8×10^6
33.	E.63.	16	*	64	64	7×10^6
34.	E.67.	16	*	64	64	7×10^6
35.	E.74.	16	32	64	64	7×10^6
36.	E.80.	64	64	>64	>64	7×10^6
37.	E.83.	16	64	>64	>64	7×10^6
J.	$H_{37}R_v$	8	16	32	64	8×10^6
38.	E.81.	16	32	64	64	4×10^6
39.	E.82.	8	16	32	64	7×10^6
40.	E.9.	16	32	32	64	7×10^6
K.	$H_{37}R_v$	8	16	32	64	8×10^6
41.	E.79.	16	32	32	64	8×10^6
42.	E.8.	16	32	32	64	5×10^6
43.	E.7.	16	32	32	*	7×10^6
44.	E.6.	64	64	>64	>64	5×10^6
L.	$H_{37}R_v$	16	32	32	>64	4×10^5
45.	E.5.	16	16	32	64	7×10^6
46.	E.2.	16	16	32	64	7×10^6
47.	E.85.	16	16	32	64	7×10^6
48.	E.84.	16	32	32	64	7×10^6
M.	$H_{37}R_v$	8	16	32	32	5×10^6

Experiments were carried out in triplicates.

* indicates atypical or uncharacteristic TB susceptibility pattern.

2.2. Cycloserine

Cycloserine (product number C 6880 – 1 G) was purchased directly from Sigma-Aldrich Quimica, S.A. (Steinheim Germany). Aqueous solutions (pH 10) were prepared on the day of performing MIC experiments using the method recommended by Sigma (2008). The protocol involved making a 100 X concentrated 64 µg/mL cycloserine stock solution by dissolving the required amount in sodium carbonate (Na_2CO_3) solution (pH 10).

The cycloserine solution was subsequently filter-sterilised using a 0.22 µm sterile bell filter (Sarstedst, Numbrecht, Germany).

2.3. MIC Determination

Isolates were recovered by growing in Middlebrook 7H9 broth. In order to obtain the logarithmic growth phase, cultures were transferred to Middlebrook 7H11 at 37 ^{o}C. When the cultures reached the third week of growth, they were suspended in individual tubes containing 4.5 mL phosphate buffered saline (PBS), 0.05 % Tween 80 and 4 – 6 glass beads. The tubes were vortexed for 5 minutes and allowed to settle for approximately 45 minutes. Following this, the upper supernatant was aspirated and adjusted to a McFarland standard of 1 (10^7 colony forming units/mL) turbidity/density (National Committee for Clinical Laboratory Standard, 2002) by using sterile triple distilled water. Colony counts were performed on the respective isolates to determine the CFU/mL.

The broth micro-dilution procedure was performed in 24-well tissue culture plates. Middlebrook 7H9 broth (supplemented with 10% oleic acid-albumin-dextrose-catalase (OADC)) was adjusted to pH 7.2. Next, the three 64 µg/mL-labelled wells on each of the plates, were treated with 1800 µL of broth and 20 µL of cycloserine. Wells that contained 64 µg/ml of cycloserine (in triplicates) were two-fold diluted down to 1 µg/mL. Plate wells without cycloserine served as drug-free controls.

Control strain $H_{37}R_v$ was used whenever an isolate or a set of isolates were being tested. An inoculum of 100 µL of 10^5 CFU/mL of TB culture was used for each well of the plate (please note that the inoculum shown in the appendix represents the CFU/mL after cycloserine had been administrated and the plates were incubated for 1 – 4 weeks). The plates were incubated for 28 days. Wells were examined at 7, 14, 21 and 28 days in order to determine TB isolates' susceptibility trends. The MIC was determined after 21 days. The MIC of cycloserine, in this study, was defined as the lowest concentration which completely inhibits the growth of MDR-TB isolates at a pH of 7.2. This definition was used to interpret susceptibility results.

2.4. Calculation of Colony Counts

Colony counts were calculated for the 10^{-3} (dilution factor) plate cultures. This was performed to determine the number of colonies present at this dilution of culture (i.e., the number of CFU/mL after establishment of the growth logarithmic phase).

3. Results and Discussion

Mycobacterium tuberculosis-infected patients are treated with first-, second-, and third-line TB drugs. However, to optimise the dosage of the drugs in regard to toxicity of specific drugs given, TB-infected patients are put on combination therapies so that the high toxicity of drugs in the serum of patients is minimised. Singh (2013) reported a case in which TB drugs can become unstable in the serum of patients, and in the case of cycloserine-treated patients, combination therapies fail to apprehend the onset of adverse effects associated with the nervous system in particular. In addition, Wolinsky (1993) confirmed that cycloserine can potentiate hypersensitivity complications in TB-infected patients.

In this study, 48 MDR-TB isolates were treated with 5 different cycloserine concentrations (8, 16, 32, 64, and > 64 µg/mL). Cycloserine minimum inhibitory concentration was determined in a 7H9 broth-based system by monitoring TB growth in 7 day intervals (7, 14, 21, 28). The response of TB isolates to the different concentrations of cycloserine was monitored relative to the control strain, $H_{37}R_v$, and the susceptibility patterns and differences among the different sets of isolates tested on different days were noted and reported. Only one isolate [E.35.] out of the 48 tested isolates had an MIC of 16 µg/mL on the 21st day and this MIC did not correlate with the MIC of the control strain (32 µg/mL). This difference was significant as it indicated that the experiments were successfully standardised relative to susceptible control strain isolate. Similarly 74 % ($^{14}/_{19}$) of the isolates that were static at an MIC of 64 µg/mL of cycloserine, did not correlate with the MIC of the $H_{37}R_v$ strain (32 µg/mL). Those 14 isolates are presented in Table 1 (E.12., 18, 22, 23, 54, 24, 26, 14, 66, 58, 63, 58, 63, 67, 74, 81). The remaining 5 isolates ($^{5}/_{19}$) were inhibited at 64 µg/mL of cycloserine in conjunction with their respectively tested $H_{37}R_v$ strains per reported sets. These isolates were: E.40., 31, 41, 65 and 24. Among 15 % ($^{7}/_{48}$) of MDR-TB isolates that were inhibited by cycloserine at a concentration of more than 64 µg/mL, all had MICs that did not correlate with the MIC of $H_{37}R_v$, but with a greater variability in the MIC of $H_{37}R_v$ in each set of tested MDR-TB isolates. Isolates E.15., 47, 53, and 71 exhibited a static effect in multiplication when they were exposed to a cycloserine concentration of greater than 64 µg/ml, while the $H_{37}R_v$ strain was static at a reported MIC of 64 µg/mL. In contrast, $H_{37}R_v$ was inhibited at a concentration of 32 µg/mL, while cycloserine was bacteriostatic to isolates E.80, 83, and 6 at >64 µg/mL. The growth of the 21 isolates that had been inhibited by a cycloserine concentration of 32 µg/mL was in correlation with the MIC of $H_{37}R_v$ on their respective testing days, and in their tested sets. These isolates are also shown in Table 1: E.25., 28, 30, 33, 39, 51, 29, 36, 70, 55, 60, 10, 8, 2, 9, 79, 8, 7, 5, 2, 85, 84.

Table 1. Minimum Inhibitory Concentration (MIC) results of cycloserine on 48 MDR-TB isolates compared to their respective MIC results of the control, $H_{37}R_v$ on day 21.

MIC (µg/mL) (day 21)	Total no. of isolates	%	Isolates correlating with the MIC of $H_{37}R_v$	%	Isolates not correlating with the MIC of $H_{37}R_v$	%
8	0	0	n/a	n/a	n/a	n/a
16	1	2	0	0	[35]	2
32	21	48	[25, 28, 30]; [33, 39, 51, 29]; [36,70]; [55, 60, 10]; [55, 60, 10]; [82, 9]; [79, 8, 7*]; [5, 2, 85,84]	100	0	0
64	19	40	[40]; [31,41]; [65, 24]	26	[12, 18, 22, 23]; [54]; [24, 26, 14]; [66, 58]; [63, 67, 74]; [81]	74
>64	7	15	0	0	[15, 47]; [53]; [71]; [80, 83]; [6]	100

*atypical growth pattern

n/a: not applicable

Brackets [ ] denote isolates tested on the same day.

Of the 21 conforming MDR-TB isolates, isolate E.7 exhibited an atypical susceptibility pattern after it had been exposed to cycloserine. It was classified as atypical because on the day of reading the MIC, growth was observed in the wells that contained 16 µg/mL and 64 µg/mL of cycloserine. Furthermore, this isolate had been susceptible to the same concentration of cycloserine (32 µg/mL) after 14 days of incubation (as the MIC read date). However, the MIC reading was invalid due to finding growth in the tissue-culture plate after 21 days. This was not ascribed to inappropriate colony counts as reported in Singh (2013) because other MDR-TB isolates had valid cycloserine MIC readings with the same colony count number as this isolate (7×10^6 CFU/mL).

Those isolates, including $H_{37}R_v$ that were positively inhibited by cycloserine with a CFU/mL of 7×10^6 are: E.22., 33, 35, 39, 29, 40, 41, 17, 53, 24, 14, 71, 63, 67, 74, 80, 83, 82, 9, 5, 2, 85 and 84. This atypical result was not attributed to any pipetting errors of cycloserine into the tissue-culture plate because all of the MIC results were noted down in triplicates (Singh, 2013) as presented in Table 1. Petrini and Hoffner (1999) and Singh (2012) provide a possible explanation to such a result. These authors suggested that by genotypically testing this isolate, one may obtain an idea into its genetic profile since resistance-acquisition in tuberculosis is not attributed to plasmid insertion of resistance genes like in *Haemophilus ducreyi*, for example, but due to genetic mutations that are induced by the toxicity of the administered tuberculosis drugs (or agents) (Petrini and Hoffner, 1999; Singh, 2012, 2013). Therefore, such clinical isolates can possibly have increased virulence and result in increased TB spread, infection and disease to others (Singh, 2013). From Table 2, it is evident that this study presents many different scenarios for reporting and interpreting minimum inhibitory concentration results in relation to MDR-TB clinical isolates. This table presents uncharacteristic isolates (E.21, 63 and 67), apart from the atypical isolate E.7, and these isolates highlight the lack of confidence that one might get from relying on MIC values obtained on day 21. Since those isolates showed increased growth by the second week of the experiment, the MIC of cycloserine at day 21 (64 µg/mL) cannot be reported with reliability. Although the fourth week MIC reading was akin to the third week reading, it is uncertain whether the second week reading could have been the same. It's for that reason that this study reported these readings as MICs for the mentioned isolates. Pipetting errors and inappropriate colony counts are not attributable to these uncharacteristic results as previously discussed and mentioned in Singh (2012, 2013).

Table 2. Isolates showing atypical and uncharacteristic TB growth inhibition in the MIC study

Isolate Number	Minimum Inhibitory Concentration (MIC)				Inoculum CFU/mL
	Day 7	Day 14	Day 21	Day 28	
21	16	*	64	64	1×10^6
63	16	*	64	64	7×10^6
67	16	*	64	64	7×10^6
7	16	32	32	*	7×10^6

* indicates atypical or uncharacteristic growth pattern.

The MDR-TB isolates in this study were tested in sets and the MIC of cycloserine for each isolate in the set was reported relative to $H_{37}R_v$. Although isolate sets were tested for susceptibility to cycloserine, the MIC results obtained for all 48 isolates did not have much variability. This, however, is not acceptable since each MDR-TB-infected patient would have been on a different treatment regimen. Therefore as stressed in Singh (2013), the complete treatment profile of patients require assessment in order to accurately interpret the clinical isolates' cycloserine susceptibility patterns. Fourteen out of the 48 isolates (29 %; E.12., 28, 39, 41, 36, 70, 55, 60, 10, 9, 79, 8, 7, 84) showed a single shift in their MIC susceptibility pattern for cycloserine from day 7 to 14 to 21, while the remaining 71 % had susceptibility patterns whereby the MIC value were repeated (at least) two times across the four weeks. The latter includes isolate E.7, which was classified as being atypical. Of these 14 isolates, only 2 isolates (E.28 and 41) had day 28 susceptibility readings that correlated with day 21 MIC values. Both of these isolates were present in the broth-based system at $8 \times 10^6 - 7 \times 10^6$ CFU/mL. However, it was deduced that when MDR-TB isolates were present at a concentration of between $8 \times 10^6 - 1 \times 10^7$ CFU/mL in the broth-based system, the concentration of cycloserine that induced tuberculosis growth inhibition was 64 µg/ml or more, with the exceptions of isolates E.66, 81, 68, and 6, because those isolates were present below this colony

count number in the experiments and would not have been considered ideal according to the McFarland standard that was used (Singh, 2012).

MDR-TB isolates E.66 and 81 were inhibited by cycloserine at concentration of 64 µg/ml though the inoculums used were low (2×10^6 and 4×10^6 CFU/mL, respectively). In contrast, although isolate E.6 was inoculated at 5×10^6 CFU/mL, a far greater concentration of cycloserine was required to inhibit its multiplication (>64 µg/mL). Isolates E.12, 39, 41, 9 and 7 were inoculated at 7×10^6 CFU/mL, but bacterial growth was inhibited at different cycloserine concentrations. Isolates E.12 and 41 were inhibited at a cycloserine concentration of more than 64 µg/mL, while the remaining 3 isolates were inhibited at 32 µg/mL. Isolates E.36, 70, 55, 60, and 10 exhibited the same cycloserine susceptibility pattern across the four weeks and were inoculated into 7H9 at 1×10^7 CFU/mL. Although this was considered a significant discovery, other isolates (E.28., 39, 9, 79, 8, 84) were found to have the same cycloserine susceptibility pattern, but used different mycobacterium inoculum counts. Singh (2012, 2013) reported and commented on this finding and has raised the question of 'at what CFU/mL would performing MIC tests be optimal at, if such an optimal exists?' The MIC results for isolates E.36, 70, 55, 60, and 10, further reiterates the question put forth by Singh (2012, 2013).

Cell counting was used to optimise and standardise the protocols used in performing drug susceptibility tests, such as the determination of (MIC) values, by providing uniform colony counts amongst multiple experiments (Singh, 2013). However, with the patients being on different treatment regimens, optimising the susceptibility on the basis of colony counts alone is not sufficient, especially since the antibiogram of the isolates is not available, as is the case in the present study. Therefore, it's probable that conclusions from drug susceptibility tests cannot be sufficiently made by simply relying on CFU/mL as an indicator, because it is possible for some (or all) patients to be on combination therapies or utilising other forms of treatment options (Singh, 2012, 2013; personal writing, 2014).

In this study, although colony counts were important for measuring MICs and/or drug susceptibility tests, it was found that the inhibition of the bacterial multiplication machinery by various cycloserine concentrations, was not influenced by the amount of bacteria being used (reviewed in Singh, 2013). For example, $H_{37}R_v$ for isolates E.36, 70 and 54 showed an MIC of 32 µg/mL on day 21. The inoculum of the $H_{37}R_v$ strain for these 3 MDR-TB isolates tested was 4×10^6 CFU/mL (i.e. relatively low). It's possible that the persistence of the MIC value after 21 days was due to the low colony count numbers compared to the concentrations of $H_{37}R_v$ used in other experiments, except for isolates E.24., 14, and 66.

The $H_{37}R_v$ isolates that were used per set of MDR-TB isolates are shown in order of ascending inoculum counts in Table 3 (3×10^6 – 8×10^6 CFU/mL). Though a proportional relationship between TB colony forming units and the amount of cycloserine administered was expected, in this study, this relationship was not apparent because experiments involving similar $H_{37}R_v$ inoculums unexpectedly resulted in different cycloserine MIC values (see experiments involving sets B, C, D, and M). It was also found that the control strain $H_{37}R_v$ used in sets K, F, L and M, exhibited the same cycloserine susceptibility pattern across four weeks with a precise cycloserine MIC value being reached after the second week. The MICs of the control strain used in sets K, E, F, C, D, M and I, were considered feasible because the concentration of cycloserine that induced growth inhibition after 3 weeks was identical to that of the fourth week. These MIC readings increased the internal and external validity of the study as stated in Singh (2013). An exception was the control strain used in set B, because it had an MIC value identical to the reported cycloserine concentration after the first week of experimentation.

Table 3. $H_{37}R_v$ isolates with the same cycloserine inhibitor concentrations for days 21 and days 28. (arranged in ascending order of colony counts)

Inoculum CFU/mL	Control Strain Set	Day 7	Day 14	Day 21	Day 28
3×10^6	K	8	16	32	32
4×10^6	E	16	32	64	64
4×10^6	F	8	16	32	32
5×10^6	B	8	32	32	32
5×10^6	C	8	16	32	32
5×10^6	D	16	32	64	64
5×10^6	M	8	16	32	32
8×10^6	I	16	32	64	64

The McFarland standard is a turbidity standard used to measure out a particular concentration of microorganism required in an experimental study (Singh, 2013). It has been reported that a dilution factor of 10^4 (starting from 10^4) would provide a working concentration of 1×10^7 CFU/mL (Singh, 2013), but other studies used a dilution factor of 10^3. Control strain of sets K, F, C and M, exhibited the same susceptibility profiles, while those in sets E, D and I, exhibited the same susceptibility profiles, but at variable inoculum CFU/mL. Hence, although the inoculums for the controls strains in sets C, D, and M was 5×10^6 CFU/mL, the susceptibility pattern of the control strain in set D mimicked that of E and I, which had control strain inoculums of 7H9 at 4×10^6 and 8×10^6 CFU/mL respectively.

Researchers and others may find the MIC readings of the MDR-TB clinical isolates to be obscure when they are compared with that of the $H_{37}R_v$ strain, but as explained by Singh (2013), such MIC values are of value and of universal importance. Isolate E.6. was present in the wells of the tissue-culture plate at the same concentration as E and F. The susceptibility profiles of the 3 isolates showed a single shift in the cycloserine inhibitory potential, but the MIC at which the growth of each isolate was inhibited, was different. In conclusion, there was no established link between the established MIC readings and the colony counts of each isolate (Singh, 2012, 2013).

A significant finding of this study was that $H_{37}R_v$ isolates in sets A, L, and B (Table 4) had MIC readings

that did not strongly correlate with the isolate sets tested on their respective test days. Since the MIC readings of the mentioned isolates changed on the 28th day, those isolates probably required genotypic testing similar to what was suggested for the atypical isolate 7. Of these 3 isolates, isolate in set B exhibited the most accurate inhibitory growth effect against cycloserine, because it retained its cycloserine MIC value (32 µg/mL) after 21 days. In contrast, control strain in sets A and C required a much higher dosage of cycloserine to induce their inhibitory effect because these isolates did not retain their MIC values after the third week, even though the MIC value may or may not be identical after 14th day. The cycloserine MIC values of 64 and >64 µg/mL for control strains in sets A and L, indicated that the isolates were either too resistant to the administered concentrations, or they were not susceptible to the administered concentrations at that given point in time. Furthermore, no established links between the MDR-TB colony counts and the MIC patterns between these 3 isolates (A, L, and B) were found, and this is postulated to be true for all the isolates (including $H_{37}R_v$), that were tested. The control strain in sets C, D, E, F, G, H, I, J, K, L, M, and N, had well-established MIC results since the inhibitory cycloserine concentrations (day 21) were akin to the MIC reading after 3 weeks, and were different from the cycloserine inhibitory concentrations after 14 days.

Table 4. $H_{37}R_v$ isolates showing poor representation MIC results when administered with cycloserine between days 7 and 14 and days 14 and 21.

Inoculum CFU/mL	Key	Day 7	Day 14	Day 21	Day 28
1×10^6	A	16	32	32	64
4×10^6	L	16	32	32	>64
5×10^6	B	8	32	32	32

Due to the absence of information regarding the treatment regimens being used at the time the 48 MDR-TB isolates were obtained, the unusual MIC results of cycloserine on specific clinical isolates (such as E.7) cannot be explained with confidence (Singh, 2013). Furthermore, individual TB-infected patients often present themselves at different stages of tuberculosis infection for treatment, and may be falsely recognised as MDR-TB during drug susceptible laboratory tests (Singh, 2012, 2013). In the present study, this could have been the reason for the cycloserine susceptibility patterns obtained for isolates E.71., 80, 83, and 6. The colony forming units for E.6. was different compared to the other 3 tested isolates. Alexander and Strete (2001) has further suggested that MIC results this high (64 or >64 µg/mL) could be attributed to the immune-compromised state of the patient. Singh (2013) emphasises that this could have been due to late tuberculosis treatment and other allied problems like financial, economic, social and sexual reasons (the introverted state of the infectious disease). Furthermore, these patients could have also been shy to confront a physician, specialist or consultant because of additional problems like AIDS, HIV co-infection and other sexually transmitted diseases.

This study was also significant because many of the MDR-TB isolates were found to be susceptible to cycloserine at similar inhibitory concentrations for the first two weeks. Isolates E.18., 22, 23, 30, 33, 15, 51, 29, 15, 47, 31, 41, 31, 41, 24, 26, 71, 80, 6, 5, 2 and 85 exhibited this characteristic (Appendix). Although these MIC values did not conclude anything about the patient's resistance to cycloserine in comparison to the MIC values after 3 weeks, reporting them is an advantage as it confirms that the tested MDR-TB isolates had different susceptibilities to cycloserine. Moreover, the increase in MIC value after 14 days, indicated a mycobacterial shift of growth inhibition to a higher concentration of the drug (Singh, 2013).

Isolate E.83 (Appendix) had a very interesting susceptibility pattern to cycloserine across the four week susceptibility period. This isolate initially required 16 µg/mL of cycloserine to slow its multiplication down before one week exposure, and a 4-fold concentration by the 14th day. Singh (2013) suggests that this could have been due to an increased resistance of E.83. to cycloserine due to genetic mutations induced by exposure to cycloserine early in the experiment.

4. Conclusion

This study demonstrated the absence of a relationship between cycloserine MIC values of the TB isolates and the inoculum CFU/mL used. This study might serve as an important 'reference' to minimising the toxic effects of cycloserine so that it's prescription as a TB drug to patients can be safe and reliable, in that it will not have an effect on the nervous system and other hypersensitive complications. Generally a blood cycloserine concentration of 30 mg/L would cause these complications, but in this study, the concentration of cycloserine that caused growth inhibition of the MDR-TB isolates were variable and at a much lower concentration compared to this value. The results of this study would allow for future studies that focus on maintaining effective doses of cycloserine, while concurrently minimising its toxicity. The results of those studies would form an important step in the wider use of this important second-line TB drug.

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