

Activity of rifampicin and linezolid combination in *Mycobacterium tuberculosis*



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ABSTRACT

Background: Linezolid (LZD) is not commonly used for treating tuberculosis (TB), but in some patients with drug-resistant TB it is being used. However, the *in vitro* LZD activity, in combination with rifampicin (RIF) against *Mycobacterium tuberculosis* has not been fully elucidated.

Aims: The aim of this study was to evaluate the *in vitro* activity of RIF/LZD combination against *M. tuberculosis* clinical isolates.

Materials and methods: The activity of the RIF/LZD combination was firstly determined in *M. tuberculosis* H₃₇Rv, 14 susceptible, 9 isoniazid nonresistant and 14 multi-drug resistant (MDR) *M. tuberculosis* clinical isolates by modified checkerboard assay, Resazurin Drugs Combination Microtiter Assay (REDCA). After, the Time Kill Curve Assay, at 0.5 × MIC of drugs, in combination and alone, was performed in *M. tuberculosis* H₃₇Rv and 8 (20.5%) of those clinical isolates, which the RIF/LZD combination showed to have synergistic effect by the checkerboard assay.

Results and conclusion: By Time Kill Curve Assay, we could observe in *M. tuberculosis* H₃₇Rv and susceptible isolates, that LZD alone, at sub inhibitory concentration, has poor effect on the bacillus death. In some cases, the bacillus growth stayed constant while in others showed regrowth at the eighth day of drug exposure. RIF alone exhibits potent concentration-dependent bactericidal activity, and was strongly dependent by the drug exposure time. The RIF/LZD combination accomplished a bacteriostatic effect in the reference strain and susceptible isolates. For the RIF resistant isolates, the RIF/LZD combination did not enhance the effect in killing bacillus. In this sense, additional, *in vitro* and *in vivo* studies are needed to evaluate the effect of RIF/LZD combination in order to better understand the adjunctive action of LZD in the treatment of TB and prevent the emergence of mutants with resistance to the available anti-TB drugs.

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1. Introduction

The emergence of drug-resistant tuberculosis (TB), especially multi-drug resistant TB (MDR-TB) and extensively drug-resistant

TB (XDR-TB), has been a major concern for the control of TB epidemic worldwide [1].

At present, the number of available drugs for treatment of drug-resistant TB is limited and requires the use of second-line drugs, which are more expensive, less effective, more toxic, and require prolonged time of treatment. As the discovery of new therapeutic agents has had few effective proposals, some drugs, already available for the treatment of infectious diseases caused by other

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bacterial species, are being redirected to the treatment of drug-resistant TB. In this sense, clinical studies on using linezolid, rifampentine, moxifloxacin, gatifloxacin and others are undergoing [2,3].

Linezolid (LZD), a drug pertaining to the oxazolidinone class, was approved by the Food and Drug Administration (FDA) in 2000 for the treatment of skin and soft tissue infection, pneumonia and other infections caused by Gram-positive bacteria that are resistant to several drugs, which include vancomycin-resistant enterococci [4].

LZD showed to be highly active, *in vivo* [5] and *in vitro* [6], against *Mycobacterium tuberculosis*, and in some rapidly [7,8] and slowly [9] growing non-tuberculous mycobacteria. Although, LZD is not commonly used for treatment of TB, it has been used as an alternative for treating patients with drug-resistant TB to most commonly used anti-TB drugs [10]. However, the *in vitro* activity of LZD alone or in combination with other drugs against *M. tuberculosis* has not been fully elucidated yet [11].

Considering the promising results obtained in clinical practice by using LZD in treatment of patients with highly drug-resistant TB, which may be an important therapeutic option, the aim of our study was to evaluate the *in vitro* activity of RIF/LZD combination against *M. tuberculosis* clinical isolates by a modified checkerboard method, the Resazurin Drugs Combination Microtiter Assay (REDCA) and Time Kill Curve Assay. The selection of RIF for the study was based on its potent bactericidal effect, which plays a

special role in the reduction of drug-resistant TB treatment time. In addition, the resistance to RIF submits the patient to a longer treatment with more toxic drugs. The study was conducted in two steps: Firstly, the REDCA was carried out to investigate RIF/LZD synergism or antagonism. After, the Time Kill Curve Assay was carried out, in isolates where the RIF/LZD combination showed synergistic effect, to provide a dynamic picture of RIF/LZD combination action and interaction over time.

2. Materials and methods

2.1. Bacterial samples

M. tuberculosis H₃₇Rv (ATCC 27294), and 37 *M. tuberculosis* clinical isolates (14 susceptible, 9 isoniazid (INH)-monoresistant and 14 MDR, previously tested for susceptibility, by the proportion method on Lowenstein-Jensen medium [12] and genotyped by Mycobacterial Interspersed Repetitive Units (MIRU) [13] and Spoligotyping [14] were studied. The reference strain and clinical isolates were firstly grown in Middlebrook 7H9 medium (Difco Laboratories, Detroit, MI, USA) supplemented with oleic acid, bovine serum albumin, dextrose and catalase (OADC) enrichment (BBL/Becton–Dickinson, Sparks, MD, USA) added with 0.2% glycerol (v/v) and 0.025% tween 80 (v/v) for 15 days at 35–37 °C.

Table 1

MIC of rifampicin (RIF) and linezolid (LZD) and Fractional Inhibitory Concentration Index (FICI) of RIF/LZD combination against reference strain *Mycobacterium tuberculosis* H₃₇Rv and clinical isolates.

Isolates	Spoligotyping	MIRU	Susceptibility profile ^a	MIC (µg/ml)		FICI RIF/LZD
				RIF	LZD	
H37Rv	NP	NP	Susceptible	0.125	0.5	0.250
14	777777777760771	324125153321	Susceptible	0.004	0.5	0.750
20	77777607760771	224221143223	Susceptible	0.03	0.125	1.500
23	37777607760771	123226143321	Susceptible	0.125	0.125	0.98
25	37777607760771	224226143321	Susceptible	0.06	0.25	2
46	77777774020771	224326132323	Susceptible	0.03	0.5	0.370
50	77777607560771	124326132222	Susceptible	0.004	0.5	2
51	77777607760731	124326132324	Susceptible	0.125	0.25	2
57	57777607760771	123326132321	Susceptible	0.03	0.25	2
60	37777607760771	224236152321	Susceptible	0.125	0.25	0.750
65	77777607760731	125336543324	Susceptible	0.06	0.125	1.020
69A	77777777720771	NP	Susceptible	0.125	0.5	0.306
97	77777777760731	124306143324	Susceptible	0.06	0.25	2
25252	00000007760771	NP	Susceptible	0.25	0.25	1
3	NP	124326163324	INH	0.004	0.5	2
12	NP	324326143324	INH	0.03	0.125	2
34	NP	12222153321	INH	0.008	0.125	1.500
43	NP	124326143324	INH	0.125	0.5	0.047
48	NP	223326132424	INH	0.06	0.25	0.760
52	NP	224313132324	INH	0.06	0.125	1.020
91	677737607760771	NP	INH	0.06	0.5	0.760
1193	77777777720771	NP	INH	0.06	0.25	0.760
4250	77777777760710	NP	INH	0.125	0.50	0.258
18	776177607760771	224326153324	INH/RIF/EMB	125	0.5	0.375
19	776177607760771	224327153324	INH/RIF/EMB	31.25	0.5	1
40A	77777607760771	NP	INH/RIF	250	0.5	1
64A	677737607760771	124325163322	INH/RIF	125	0.125	1
68A	776177607760771	NP	INH/RIF/PZA	62.5	0.25	0.750
71A	77777777720771	225313153323	INH/RIF/PZA	12	0.25	0.625
73A	677737607760771	224225123321	INH/RIF/PZA	4	0.125	0.750
109	776177607760771	224326153325	INH/RIF	62.5	0.5	1
3408	00000000003771	NP	INH/RIF/EMB/STR	64	0.125	1.500
3614	677737607760771	224225163321	INH/RIF/EMB/STR/ETH	8	0.125	1
485	677737607600771	NP	INH/RIF/STR	62.5	0.25	1
309	776177607760771	NP	INH/RIF/STR	250	0.25	0.312
251-S	37777607760771	NP	INH/RIF	8	0.5	0.749
97-S	77777607560771	NP	INH/RIF/STR	8	2.5	0.313

^a INH: resistance to isoniazid; RIF: resistance to rifampicin; EMB: resistance to ethambutol, PZA: resistance to pyrazinamide; STR: resistance to streptomycin, ETH: resistance to ethionamide, Numbers in bold are synergism, NP: Not performed.

2.2. Minimal inhibitory concentration (MIC)

The MICs of LZD (Zyvox[®], Pfizer, Brazil) and RIF (Sigma, St Louis, USA) for *M. tuberculosis* H₃₇Rv and the clinical isolates were performed three times on different days by Resazurin Microtiter Plate Assay (REMA) as described by Palomino et al. [15]. Bacterial growth and sterility controls were performed in all assays. The MIC was assessed visually by color change from blue to pink, which indicates reduction of resazurin by bacterial growth.

2.3. Resazurin Drugs Combination Microtiter Assay – REDCA

The antimicrobial activity of RIF/LZD combination was performed three times on different days by REDCA [16] in microtiter plates with 96 wells. The fractional inhibitory concentration index (FICI) was used to evaluate the combination effect, and the results were interpreted as synergism (FICI ≤ 0.5), no interaction (FICI > 0.5–4) and antagonism (FICI > 4) [17].

2.4. Time Kill study

The Time Kill Curve Assay was performed three times on different days as described by Limoncu et al. [18], with modifications for drug combinations in *M. tuberculosis* H₃₇Rv and clinical isolates, which previously showed synergistic effect by REDCA (46, 69A, 43, 4250, 18, 309, 97-S). LZD, RIF and RIF/LZD drug combination were added to each standardized mycobacterial suspension to achieve 0.5 × MIC of drugs previously determined for each isolate. A growth control containing no drug was also included. The cultures were incubated at 35–37 °C under shaking at 96 rpm for 8 days. Aliquots (100 µl) of each mycobacterial growth at initial, 3, 5 and 8 days, were serially diluted in phosphate buffered saline (PBS) to 10⁻¹, 10⁻³ and 10⁻⁵ and plated on Middlebrook 7H11 agar (Difco Laboratories, Detroit, MI, USA) supplemented with OADC. All plates were incubated at 35–37 °C for 21 days at normal atmosphere and after the colony forming units (CFUs) counting was carried out.

3. Results

The MIC of RIF for susceptible (including INH monoresistant isolates) and RIF resistant *M. tuberculosis* clinical isolates ranged from 0.004 to 0.25 µg/ml and 4–250 µg/ml, respectively. For LZD, the MIC ranged from 0.125 to 0.5 µg/ml for susceptible and 0.125–2.5 µg/ml for RIF resistant clinical isolates (Table 1).

By REDCA, the synergistic effect of RIF/LZD combination (FICI ≤ 0.5) was observed in *M. tuberculosis* H₃₇Rv and 8 (20.5%) clinical isolates. From these, 3 (37.5%) isolates were susceptible, 2 (25.0%) INH monoresistant and 3 (37.5%) MDR. No antagonism with the drugs combination tested (FICI > 4) was observed (Table 1).

The Time Kill curve assay for the H₃₇Rv, 4 RIF susceptible (46, 43, 4250 and 69A) and 3 RIF resistant (18, 309 and 97-S) isolates are shown in Fig. 1. For the RIF susceptible isolates the RIF alone led to a reduction of bacterial concentration, starting at the third day, with 0.5 × MIC concentration. No significant reduction of bacterial concentration was observed in RIF resistant isolates. The LZD alone showed reduction of bacilli concentration, in both susceptible and resistant isolates. However, that reduction was lower than the obtained with RIF alone. The RIF/LZD combination showed similar action to the one of RIF alone in susceptible and resistant isolate.

4. Discussion

It is understandable that a multidrug therapy is essential for the treatment of TB, and is a way to avoid the emergence of resistant strains. Until new anti-TB drugs are developed to combat the drug-

resistant TB, novel combinations with the existing drugs are perhaps the best recourse in such scenario. Due to the varied resistant genotypes that may be exhibited by *M. tuberculosis* isolates, the selection of empirical drugs combination treatment is difficult because of the lack of common effective drugs combination for all resistant isolates.

Each new drug candidate or inclusion of a drug already used against other microorganism to be included for anti-TB therapy, needs to be assessed on its interactions with the classical anti-TB drugs currently used. This is essential in order to visualize the potential of interaction among the drugs and avoid possible antagonisms and then result in selection of resistant mutant bacilli.

To our knowledge, there was no extensive study on the use of LZD for treatment of MDR-TB or XDR-TB and, also, studies that evaluated the *in vitro* activity of LZD individually or in combination with anti-TB drugs in *M. tuberculosis* clinical isolates. Based on this, our study was conducted specifically to have a first insight about the *in vitro* activity of LZD and RIF alone and in combination against local susceptible and with decreased susceptibility to RIF *M. tuberculosis* clinical isolates.

The MIC values of LZD alone for *M. tuberculosis*, observed in this study (0.125–2.5 µg/ml), were similar to those observed in studies carried out by Guna et al. [8] (0.125–4.0 µg/ml), Ermercan et al. [19] (0.6–1.0 µg/ml), Alffenaar et al. [20] (≤0.25–1.0 µg/ml), Alcalá et al. [6] (1.0–2.0 µg/ml) and Yang et al. [21] (0.125–0.5 µg/ml), which included MDR and extensively-drug resistant (XDR) *M. tuberculosis* isolates in their works.

Although Rodríguez Díaz et al. [22] and Coban et al. [23] found no *in vitro* synergism with RIF/LZD combination, by classic checkerboard method, in RIF-monoresistant and MDR *M. tuberculosis* isolates, in our study, by a modified checkerboard method, REDCA, which uses resazurin for reading the assay, synergism was observed in 8/39 (20.5%) of local clinical isolate. From these, 2/8 (25%) were INH-monoresistant, 3/8 (37.5%) were MDR and 3/8 (37.5%) were susceptible isolates. In relation to the RIF susceptible clinical isolates, which account for 5/8 (62.5%), our results are higher than those obtained by Rodríguez-Díaz et al. [22] that observed RIF/LZD synergism in 33% of RIF susceptible isolates.

Time Kill Curve Assays were carried out at sub inhibitory concentration (0.5 × MIC), previously determined for each drug individually and in combination in all isolates, in which synergism was previously observed by REDCA. The 0.5 × MIC fixed concentration was chosen to compare the *in vitro* killing dynamics of RIF and LZD alone and in combination by the exposition time used in the REDCA. The better action of RIF against the bacillus, among the exposition time here, was in the eighth day of exposure for RIF susceptible isolates. Our results corroborate with the literature that RIF exhibits potent activity dependent of exposure time too [24]. The RIF resistant isolates (18, 309 and 97-S) showed reduction in the bacterial inoculum concentration < 3 log₁₀ CFU/ml when exposed to RIF alone, which was expected by the RIF resistant status of the isolates.

LZD alone, at 0.5 × MIC, had very poor effect on the RIF susceptible and resistant bacilli death. In some cases, the mycobacterial growth, in the assay with LZD alone, stayed constant and other showed bacillary regrowth at the eighth day of drug exposure.

Working with RIF exposure at 0.5 × MIC, we also could observe that H₃₇Rv and the clinical isolates 46, in which both showed MIC 0.003 µg/ml for RIF, had ≥3 log₁₀ CFU/ml decrease in the colony counting, compared to the initial inoculum concentration, at 8 days of RIF exposure. The isolates 69A, 43 and 4250 also showed decrease in the colony count, at 8 days of RIF exposure, but not ≥3 log₁₀ CFU/ml as compared to the initial inoculum concentration. This may be attributed to the relatively higher RIF MICs (0.125 µg/ml) that these isolates harbored.

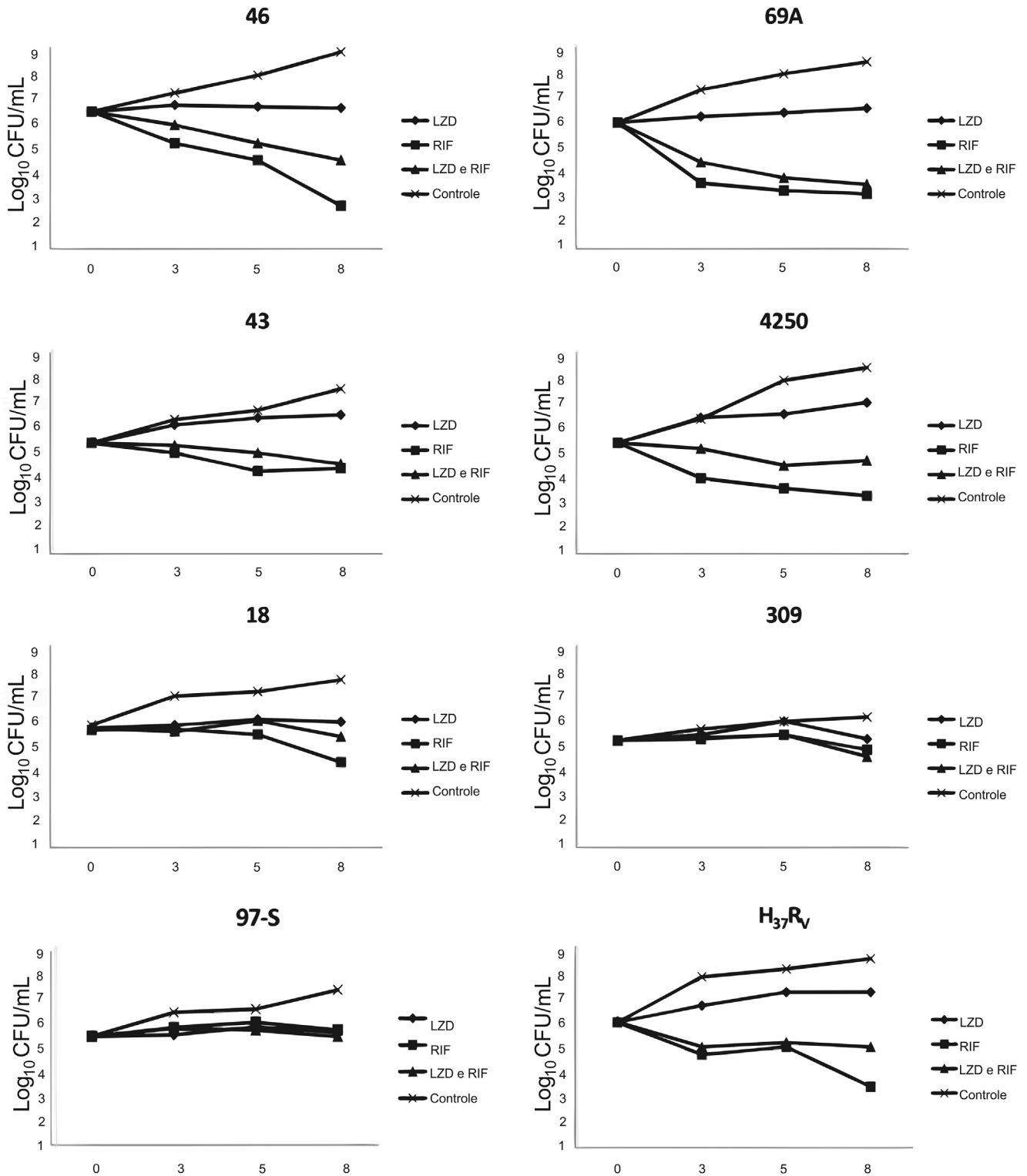


Fig. 1. Time Kill Curve of RIF susceptible (46, 43, 69A and 4250), multidrug resistant (18, 309 and 97-S) *M. tuberculosis* clinical isolates and the reference strain H₃₇R_v.

The RIF/LZD combination, at $0.5 \times \text{MIC}$, accomplished an *in vitro* bacteriostatic effect in the reference strain (decrease of $1 \log_{10}$ CFU/ml) and in the susceptible isolates (decrease of ≤ 1 to $2.46 \log_{10}$ CFU/ml). For the RIF resistant isolates (18, 309, 97-S) the RIF/LZD combination did not enhanced the effect against the bacilli, as obtained with RIF alone. However, no antagonism was observed with the RIF/LZD combination in the studied local isolates.

In clinical practice, a LZD-containing regimen to treat some patients with XDR-TB has shown excellent results in combating this form of the disease [25–27]. In these specific cases, the drugs combination, including LZD, significantly improved patients clinical symptoms by closing pulmonary cavities, accelerate expectoration and caused sputum to be acid fast bacilli negative. However, it is hardly recommended that clinicians must monitor patients for

several adverse reactions in using long-term LZD application. According to Lee et al. [27], to avoid this obstacle by the combined therapy, the dosage of 300 mg per day of LZD is appropriate to maintain drug serum level and cause fewer adverse events.

In this analyzes we must consider the mechanism of action of the two studied drugs, RIF and LZD. The two drugs have actions by inhibiting the protein synthesis in the bacillus, although by different mechanisms. LZD inhibits protein synthesis by binding to the 23S ribosomal RNA, which is a component of ribosomal 50S subunit preventing the formation of ribosome (70S), which will impair the protein synthesis. RIF, also inhibits protein synthesis, but in a prior step, by binding to the beta subunit of the RNA polymerase (RpoB), encoded by *rpoB* gene, that block the transcriptional process and consequently all the protein synthesis.

Then, depending on the causative mechanism of RIF resistance, as mutation in a defined region of the *rpoB* gene or other unknown yet, the combined therapy with LZD could be very useful for some patients, as have been reported by some clinical studies [25–27]. However, attention to carefully use this combination should be clear due to the likelihood of developing LZD and RIF resistant mutants.

The research on drugs synergism or the detection of the lack of antagonism with RIF are of paramount importance, since RIF has a key role in reducing the time for treating TB [24]. We need to analyze each situation since it is well stated that RIF has considerable side effects, which often induce cessation of treatment by some patients and can lead to selection of resistant strains. The reduction of RIF and LZD dosage by the combination might be beneficial for some patients, as it reduces the undesirable effects and thereby improves treatment adherence. It is also interesting to note that the reduction of RIF dosages may be important in the treatment of HIV/AIDS co-infected patients, in which interaction with antiretroviral leads to RIF or antiretroviral decrease in serum [1]. On the other hand the increase of RIF dosage instead of using the combination should be taken into account in particular patients with low RIF resistance, in which the resistant mechanisms is not by *rpoB*, but by another mechanism as efflux pumps.

5. Conclusion

Finally, no antagonism between RIF and LZD was observed in our study with *M. tuberculosis* isolates. However, all should bear in mind that the study was carried out *in vitro* with a limited number of local clinical isolates and only with the combination of two drugs, RIF plus LZD. Additionally, the potential genetic differences among *M. tuberculosis* isolates in response to drugs must be considered.

In this sense, additional *in vitro* and *in vivo* studies are needed to evaluate the effect of RIF/LZD or combination with other anti-TB drugs such as INH and EMB, in order to better understand the adjunctive action of LZD in the treatment of TB. Nevertheless, *in vivo* studies will have also to be conducted in order to consider the combination of LZD and anti-TB drugs as an option to prevent the emergence of mutants with resistance to the available anti-TB drugs.

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Ethical approval

Not required.

Competing interest

The authors declare that they have no competing interest.

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