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Combinatory activity of linezolid and levofloxacin with antituberculosis drugs in *Mycobacterium tuberculosis*

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ABSTRACT

Setting: The increase of multidrug and extensively drug resistant *Mycobacterium tuberculosis* strains turns the search for new tuberculosis (TB) treatment options of paramount importance.
Objective: In this sense, the present study evaluates the *in vitro* activity of isoniazid (INH)/rifampicin (RIF)/levofloxacin (LVX) and INH/RIF/linezolid (LNZ) combinations in resistant *M. tuberculosis*.
Design: The activities of the combinations were evaluated with *M. tuberculosis* $H_{37}Rv$, susceptible and 10 resistant clinical isolates by three-dimensional checkerboard. LVX and LNZ were used as the third drug at fixed ½ and ¼ minimum inhibitory concentration (MIC). INH and RIF were tested at concentrations ranging from 0.0009 µg/mL to 50 µg/mL and 0.0009 µg/mL to 800 µg/mL, respectively. The combinatorial effects were determined by the Fractional Inhibitory Concentration Index (FICI). FICI values ≤ 0.75, 0.75–4 and ≥4 were considered as synergism, indifferent and antagonism, respectively.
Results: MIC ranged from 0.03 - 6.25 µg/mL for INH, 0.008–100 µg/mL for RIF, 0.12–0.25 µg/mL for LVX and 0.25–0.5 µg/mL for LNZ in the $H_{37}Rv$ and all clinical isolates. INH/RIF/LVX and INH/RIF/LNZ synergisms were observed in 40 and 50% of the resistant *M. tuberculosis* clinical isolates and better observed for INH and RIF combined to LVX or LNZ at ¼ MIC.
Conclusion: The present study calls attention for the potential use of INH/RIF/LVX and INH/RIF/LNZ combinations in the treatment of resistant TB.

1. Introduction

Tuberculosis (TB), which has been known for centuries, continues to be one of major global public health problems. In 2015, TB ranked first in the world for death due to infectious diseases, surpassing for the first time the number of deaths caused by the immunodeficiency virus (HIV) [1].

The recommended treatment for new cases of TB consists of the isoniazid (INH), rifampicin (RIF), pyrazinamide (PZA) and ethambutol (EMB) drugs combination in the first 2 months added of 4 months of INH and RIF. Situations such as monotherapy, improper prescription of this association or the lack of patient's compliance for using this therapeutic regimen may lead to the emergence of resistant *Mycobacterium tuberculosis* to one or more drugs [1].

TB control has been severely compromised by the emergence of multidrug resistance (MDR), resistance to INH and RIF, which accounted for 480,000 new MDR-TB cases in the world in 2016 [1], and extensively drug-resistance (XDR), an MDR isolate with added resistance to a fluoroquinolone and one of the three second-line injectable drugs (amikacin, kanamycin or capreomycin) [2]. Treatment options for patients with MDR-TB and especially with XDR-TB are very limited [2].

In the search for new TB therapy options and with lower toxicity, other drugs are being studied, such as levofloxacin (LVX), moxifloxacin,

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linezolid (LNZ), amoxicillin-clavulanate, clarithromycin, thioridazine and clofazimine [1,2]. LVX is a third generation fluoroquinolone, which has marked activity against *M. tuberculosis* compared to other drugs of the same class [3]. LNZ, which is a drug belonging to the class of oxazolidinones, has also shown to have early bactericidal activity against *M. tuberculosis* [4].

Previous two-dimensional LNZ or LVX interaction with anti-TB drugs studies in *M. tuberculosis* $H_{37}Rv$ (ATCC 27294) and clinical isolates showed synergism against the bacillus [3–5]. As the present therapy for MDR and XDR-TB uses LNZ or LVX combined to more than two anti-TB drugs and no information about these interactions exist, a better understanding on how three or more drugs combinations work in *M. tuberculosis* is of great interest. In this sense, we describe for the first time an *in vitro* study of LVX and LNZ activity combined with INH and RIF by three-dimensional checkerboard in monoresistant and MDR *M. tuberculosis* clinical isolates.

2. Material and methods

2.1. Reference strain, susceptible and resistant M. tuberculosis clinical isolates

M. tuberculosis $H_{37}Rv$ (ATCC 27294) and 11 *M. tuberculosis* clinical isolates (six MDR, three INH monoresistant, one SM monoresistant and one susceptible, from the Laboratory of Medical Bacteriology of the Universidade Estadual de Maringá), were cultured in Middlebrook 7H9 (Difco Laboratories, Detroit, USA) with Oleic Acid, Albumin, Dextrose and Catalase-OADC Enrichment (BBL/Becton-Dickinson, Sparks, MD, USA) and incubated for 15 days at 35 °C. Bacterial suspensions were standardized with McFarland No. 1 scale and diluted 1:20 in Middlebrook 7H9 supplemented with OADC to carry out Minimum Inhibitory Concentration (MIC) and drugs combinations assays. Committee approval was not necessary.

2.2. Antimicrobial agents

INH, RIF (Sigma-Aldrich, St. Louis, MO), LVX (HalexIstar Co, Goiania, GO, Brazil) and LNZ (Zyvox®, Pfizer, Brazil) were diluted according to the manufacturer's instructions. Dilutions from 50 to 0.0009 μg/mL, 800 to 0.0009 μg/mL, 8 to 0.062 μg/mL and 8 to 0.031 μg/mL were performed directly in the microplates wells for INH, RIF, LVX and LNZ, respectively.

2.3. Minimum Inhibitory Concentration (MIC)

The MIC of each drug for *M. tuberculosis* $H_{37}Rv$ and clinical isolates was determined by Resazurin Microtiter Assay Plate (REMA) as described by Palomino et al. (2002) in triplicate in different days [6]. Drugs were serially diluted with Middlebrook 7H9 supplemented with OADC in 96-well microplates. Then, 100 μL of the previously standardized bacterial inocula were added. The microplates were incubated at 35 °C in normal atmosphere for seven days. After, 30 μL of freshly prepared 0.02% resazurin (Acros, Morris Plains, NJ, USA) were added to each well and the plates were incubated again for 24 h - 48 h at 35 °C. The color change from blue to pink, by the reduction of resazurin, was considered as presence of bacterial growth. MIC was defined as the lowest concentration of the drug that inhibited the bacterial growth. The reference values considered for susceptibilities were MIC ≤ 0.25 μg/mL for INH [5,6], ≤ 0.5 μg/mL for RIF [3,6], ≤ 1 μg/mL for LVX and LNZ [5,7].

2.4. Three-dimensional checkerboard method

The *in vitro* combination assay of INH plus RIF with LVX and INH plus RIF with LNZ was evaluated in triplicate by the modified three-dimensional checkerboard method employing resazurin as mycobacterial growth indicator agent [7,8]. INH (columns in the microplate) and RIF (rows in the microplate) were two-fold serially diluted. After, LVX or LNZ, at fixed subinhibitory concentrations (½ or ¼ MIC) previously determined, were added to each well. The 1:20 McFarland No. 1 scale diluted bacterial inoculum (100 μL) was added to each well. The plates were incubated at 35 °C for seven days. After, 30 μL of freshly prepared 0.02% resazurin (Acros, Morris Plains, NJ, USA) were added to each well and the plates were incubated for 24 h - 48 h at 35 °C. Middlebrook 7H9-OADC without drugs and without bacterial inoculum and Middlebrook 7H9-OADC without drugs and with bacterial inoculum were used as Negative control and Positive control, respectively. The color change from blue to pink was considered as presence of bacterial growth.

The possible drugs combinatorial effects were determined using the mathematical model by the determination of the Fractional Inhibitory Concentration Index (FICI) = (MIC A + B + C/MIC A) + (MIC B + C + A/MIC B) + (MIC C + A + B/MIC C), where MIC A + B + C is the MIC of drug A combined with drugs B and C, MIC B + C + A is the MIC of drug B combined with drugs A and C, MIC C + A + B/MIC C is the MIC of drug C combined with drugs A and B. MIC A, MIC B and MIC C is the MIC of drug A, B and C tested alone, respectively. The interpretation of the antimicrobials combinations was performed according to Bhusal et al. (2005) [2]. The effects of the antimicrobial combinations were classified as synergistic (FICI ≤ 0.75), additive or indifferent (FICI > 0.75 to 4.0) and antagonist (FICI > 4.0) [2].

3. Results and discussion

The recommended therapy for TB is based on the use of drugs combinations and a prolonged time for killing the bacillus in its different stages of the disease development. The basic treatment for newly diagnosed TB cases adopts the combination of INH, RIF, PZA and EMB [1]. However, in situations of monoresistance, MDR-TB or XDR-TB, this therapeutic scheme does not reach the desired effect.

In some MDR-TB and XDR-TB cases, LVX or LNZ have been used as alternative drugs for treating patients with this severe form of the disease. The use of these drugs, as well as other new combinations, has given to the patients with resistant TB new chances of cure. However, their combinatorial activity with classical anti-TB drugs was not totally determined.

In our study, we observed that the combinations of INH/RIF with LVX or LNZ showed a satisfactory effect against the resistant *M. tuberculosis* clinical isolates tested, leading to a decrease in the MICs values of the two classical anti-TB drugs, INH and RIF.

A total of 50% (n = 5, BRF 23, 309, 71 A, 19 RP and 3614) of resistant *M. tuberculosis* clinical isolates showed a decrease of at least two-fold in the INH MIC when ½ MIC LVX and 50% (n = 5, BRF 7, BRF 26, 309, 71 A and 109) when ¼ MIC LVX were combined. A higher percentage of clinical isolates, such as 70% (n = 7, BRF 27, BRF 45, 309, 71 A, 109, 19 RP and 3614) and 90% (n = 9, BRF 27, BRF 7, BRF 14, BRF 23, BRF 45, 71 A, 109, 19 RP and 3614), showed decrease of at least two-fold in the RIF MIC when combined to ½ MIC LVX and ¼ MIC LVX, respectively (Table 1).

Synergistic effect among INH/RIF/LVX was observed in 40% of resistant *M. tuberculosis* clinical isolates. Rodriguez Díaz et al. (2003) [9], which performed *in vitro* two-dimensional checkerboard with LVX combined to INH or RIF, also observed synergism against susceptible and INH monoresistant *M. tuberculosis* clinical isolates. The synergistic activity of INH/RIF/LVX was observed in the clinical isolates BRF 7 (SM monoresistant), 71A (MDR), 109 (MDR) when INH and RIF were combined to ¼ MIC LVX. For the 309 (MDR) isolate, the synergistic activity was observed only with ½ MIC LVX.

Rastogi et al. (1996) [10], twenty years ago, demonstrated, in their *in vivo* study, that the addition of LVX to the standard anti-TB treatment had satisfactory effects for patients, conducting positive to negative culture conversion for more than 80% patients with XDR-TB in a