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BRIEF COMMUNICATION

# Anti-*Mycobacterium tuberculosis* activity of antituberculosis drugs and amoxicillin/clavulanate combination

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**Abstract** We report the *in vitro* drugs interaction by the resazurin drugs combination microtiter assay (REDCA) of amoxicillin (AMO)/clavulanate (CLAV) with isoniazid (INH), ethambutol (EMB), and rifampicin (RIF) against susceptible and resistant *Mycobacterium tuberculosis* isolates. The addition of AMO/CLAV to classical antituberculosis drugs should be explored as a promising alternative for the treatment of resistant tuberculosis (TB).


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## Introduction

The treatment of tuberculosis (TB) currently occurs with a specific regimen of isoniazid (INH), rifampicin (RIF), pyrazinamide (PZA), and ethambutol (EMB) combination. Failure to provide adequate medication and patient noncompliance to treatment have resulted in resistant *Mycobacterium tuberculosis*. Multidrug-resistant (MDR) and extensively drug resistant isolates have consequently emerged and caused serious problems worldwide.[1]

There are no short-term prospects for new effective antituberculosis drugs. Therefore, drugs that act on other bacteria have been empirically used for the treatment of resistant TB with promising results.

The use of well-known drugs combined with the classic antituberculosis drugs may be a valuable alternative. β-Lactam antibiotics that have inherently low toxicity are the drugs of choice for the treatment of patients with infections caused by Gram-negative and Gram-positive bacteria. Amoxicillin (AMO) exerts a bactericidal effect by binding to penicillin-binding proteins in the bacterial cell wall, inhibiting cell division, producing elongation, and lysing susceptible bacteria.[2]

*M. tuberculosis* shows intrinsic resistance to β-lactams, mainly attributed to low permeability of the bacillus cell wall and a constitutive β-lactamase that hydrolyzes most penicillins and cephalosporins.[3] However, potent *in vitro* activity against laboratory strains has been shown with the advent of potent β-lactamase inhibitors [sulbactam and clavulanate (CLAV)] and other β-lactams, such as carbapenems (imipenem and meropenem) that are not substrates for *M. tuberculosis* β-lactamase.[4]

AMO/CLAV potassium (AMO/CLAV; 1:4), a β-lactam and β-lactamase inhibitor combination, has been used for the treatment of infections caused by bacteria that are resistant to AMO, including *M. tuberculosis*, when the current treatment for TB is ineffective.[2,5] Although, previous reports *in vitro* by Gonzalo and Drobniewski[6] and *in vivo* by Chambers et al[5] showed that AMO/CLAV appears to have action against *M. tuberculosis* and is successful in the treatment of patients with resistant TB, respectively, this approach is scantly known. Our aim was to evaluate the *in vitro* activity of AMO/CLAV in combination with INH, EMB, and RIF against susceptible and resistant *M. tuberculosis* clinical isolates.

## Methods

### Bacterial samples and minimal inhibitory concentration

*M. tuberculosis* $H_{37}Rv$, 10 susceptible and 13 resistant (6 MDR) *M. tuberculosis* clinical isolates were included in the study. The minimal inhibitory concentrations (MICs) for INH, RIF, and EMB (all Sigma–Aldrich, St. Louis, MO, USA) were determined in triplicate on different days for each isolate, to confirm repeatability, using the resazurin microtiter plate assay as described elsewhere.[7] Bacterial growth, medium, and drug sterility controls were included in all assays. Isolates with MIC $\geq$ 0.25 mg/L, 0.5 mg/L, and 4 mg/L were considered resistant to INH, RIF, and EMB, respectively.[9]

### Drug interactions

Interactions between drugs were determined by the resazurin drugs combination microtiter assay (REDCA). Two fold serial dilutions from 12.5– 0.19 mg/L for AMO/CLAV (EMS, Hortolândia, SP, Brazil), 128–0.0009 mg/L for INH, 256–0.00045 mg/L for EMB, and 128–0.0312 mg/L for RIF. Afterward, 100 μL of each standardized bacterial suspension (1 McFarland turbidity scale, diluted 1:20) was added to each drug dilution. The microplates were sealed and incubated at 35°C for 7 days. Then, 30 μL of freshly prepared 0.01% (w/v) resazurin solution (Acros, Morris Plains, NJ, USA) was added to the wells, and the plates were incubated at 35°C for an additional 24–48 hours. Bacterial growth, medium, and drug sterility controls were included in all assays. A change in color from blue to pink, which reflects reduction of resazurin by bacterial metabolism, was considered bacterial growth. The fractional inhibitory concentration index (FICI) was used to evaluate synergistic effects: $FICI = (MIC\ A + B/MIC\ A) + (MIC\ B + A/MIC\ B)$. *MIC A + B* represents the MIC of drug A associated with drug B. *MIC B + A* represents the MIC of drug B associated with drug A. *MIC A* and *MIC B* represent the MIC of drugs A and B tested separately, respectively. The effects of the drug combinations were classified as synergistic (FICI $\leq$ 0.5), additive or no interaction (FICI $>$ 0.5–4), and antagonistic (FICI $>$ 4).[8]

## Results

The MICs for susceptible and resistant isolates were 0.03–0.25 mg/L and 0.5–16 mg/L, respectively, for INH, 0.007–0.25 mg/L and 2–32 mg/L, respectively, for RIF, and 0.5–2 mg/L and 4–16 mg/L, respectively, for EMB. For AMO/CLAV, the MICs ranged from 2 mg/L to 16 mg/L for all isolates (Table 1).

A synergistic effect of the AMO/CLAV + INH combination (FICI 0.187–0.312) was observed in eight (35%) isolates (2 susceptible and 6 resistant to at least INH). The AMO/CLAV + EMB combination had a synergistic effect in 19 (83%) isolates (5 resistant to EMB, INH, and RIF) with an FICI range of 0.128–0.5. The AMO/CLAV + RIF combination had a synergistic effect in 19 (83%) isolates (3 MDR) with an FICI range of 0.092–0.5. For *M. tuberculosis* $H_{37}Rv$, only the AMO/CLAV + EMB combination showed a synergistic effect (FICI 0.187; Table 1).

No antagonism between AMO/CLAV and INH, RIF, and EMB was observed.

## Discussion

The present study evaluated the *in vitro* interaction between AMO/CLAV and first-line antituberculosis drugs (INH, RIF, and EMB) against *M. tuberculosis* using REDCA, once there were limited available data on these combinations in clinical isolates.

The MICs for the reference strain, susceptible and resistant *M. tuberculosis* isolates ranged from 2 mg/mL to 16 mg/L for AMO/CLAV. These results are consistent with those obtained by Abate and Miorner,[2] but different from those obtained by Varshochi et al,[4] which reported higher

**Table 1** Susceptibility, minimal inhibitory concentration (MIC) for amoxicillin/clavulanate, isoniazid, rifampicin, and ethambutol by resazurin microtiter plate assay (REMA) and fractional inhibitory concentration index (FICI) of drugs combination by resazurin drugs combination microtiter assay (REDCA) in *Mycobacterium tuberculosis* $H_{37}Rv$ and *M. tuberculosis* clinical isolates.

| Isolates | Susceptibility | MIC mg/L | | | | FICI | | |
|---|---|---|---|---|---|---|---|---|
| | | AMO/CLAV | INH | RIF | EMB | AMO/CLAV + INH | AMO/CLAV + RIF | AMO/CLAV + EMB |
| $H_{37}Rv$ | Susceptible | 4 | 0.030 | 0.125 | 2 | 0.625 | 0.530 | **0.187** |
| 14 | Susceptible | 2 | 0.060 | 0.030 | 0.5 | 2 | **0.375** | 0.750 |
| 60 | Susceptible | 2 | 0.250 | 0.125 | 2 | **0.265** | **0.155** | **0.187** |
| 20 | Susceptible | 4 | 0.250 | 0.250 | 2 | 2 | **0.140** | **0.250** |
| 50 | Susceptible | 8 | 0.030 | 0.125 | 1 | 2 | **0.155** | **0.312** |
| TB24 | Susceptible | 4 | 0.250 | 0.015 | 1 | 0.515 | **0.5** | **0.312** |
| TB27 | Susceptible | 4 | 0.030 | 0.015 | 0.5 | 0.625 | **0.5** | 0.625 |
| 65 | Susceptible | 4 | 0.030 | 0.125 | 2 | 0.625 | **0.280** | **0.187** |
| 25 | Susceptible | 4 | 0.250 | 0.012 | 1 | 2 | **0.155** | **0.312** |
| 24 | Susceptible | 2 | 0.030 | 0.125 | 2 | **0.250** | **0.153** | **0.187** |
| 13638 | Susceptible | 4 | 0.060 | 0.007 | 2 | 0.562 | 0.750 | **0.128** |
| 4250 | INH[a] | 16 | 1 | 0.030 | 2 | 2 | **0.375** | **0.187** |
| 1193 | INH/EMB[a] | 8 | 2 | 0.030 | 8 | 2 | **0.380** | **0.312** |
| 34 | INH[a] | 8 | 1 | 0.030 | 2 | 2 | **0.375** | **0.372** |
| 3 | INH[a] | 8 | 4 | 0.125 | 2 | **0.321** | **0.280** | **0.250** |
| 1 | INH[a] | 8 | 2 | 0.125 | 2 | **0.187** | **0.155** | **0.5** |
| 51 | INH/RIF[a,b] | 2 | 1 | 2 | 1 | **0·5** | **0.188** | **0.375** |
| 43 | INH/EMB[a] | 8 | 16 | 0.125 | 16 | **0.253** | **0.092** | **0.140** |
| 52 | INH[a] | 16 | 0.5 | 0.060 | 2 | **0.312** | **0.312** | **0.141** |
| 64-A | INH/RIF[a,b] | 4 | 1 | 16 | 1 | 0.75 | **0.281** | 0.750 |
| 71-A | INH/EMB/RIF[aR*] | 2 | 2 | 32 | 8 | 2 | **0.140** | **0.250** |
| 73-A | INH/RIF[a,b] | 8 | 4 | 8 | 2 | **0.187** | 2 | 0.625 |
| 18 | INH/EMB/RIF[a,b] | 4 | 2 | 32 | 4 | 2 | 2 | **0.312** |
| 19 | INH/EMB/RIF[a,b] | 8 | 4 | 16 | | 2 | 2 | **0.312** |

[a] Resistance.
[b] Multidrug resistant isolates.
Numbers in bold are synergism.
AMO/CLAV = amoxicillin/clavulanate; EMB = ethambutol; INH = isoniazid; RIF = rifampicin.

MIC values (32–512 mg/L). Also, Hugonnet et al[9] observed an MIC >10 mg/L for AMO in the presence of 2.5 mg/L CLAV in the $H_{37}Rv$ reference strain. The differences in the MIC values from our results may be related to differences in the methodology used.

To our knowledge, no previous study has evaluated the combination of classical antituberculosis drugs, such as INH and AMO. However, studies that assessed combinations of AMO and new compounds, such as dihydromycoplanecin[10] and oleanolic acid,[11] showed promising activity against *M. tuberculosis*, mainly in resistant isolates. In our study, the AMO/CLAV + INH combination showed positive interactions in eight isolates (6 resistant to INH), reflected by low FICI values (0.187–0.312). In some of the tested isolates, decreases in the MICs were observed, but they were insufficient to be considered synergic.

The AMO/CLAV + RIF combination showed synergistic effects in 19 clinical isolates (FICI 0.140–0.380). Of these, nine were susceptible to all three antituberculosis drugs, 10 were resistant to INH, and three were MDR. This positive interaction between RIF and AMO/CLAV in MDR clinical isolates suggests that after careful clinical evaluation of the patients, there is a valuable use of this combination in cases of MDR.

RIF is important in TB treatment because it plays a key role in reducing the time of treatment, but side effects occur in some patients who use the recommended dosage.[3] Also, considering the AMO/CLAV + RIF positive interaction, a decrease in the RIF dosage may have great value in lessening the side effects that often cause the discontinuation of RIF treatment and lead to a rise in resistant strains. Additionally, we should also consider the importance of reducing the dosage of RIF in HIV/AIDS patients, in which an interaction with antiretroviral drugs leads to decrease in the serum levels of RIF and antiretrovirals.

For the AMO/CLAV + EMB combination, a synergistic effect was observed in $H_{37}Rv$ (FICI 0.187) and in 19 clinical isolates (FICI 0.128–0.375). Of these, all four EMB-resistant clinical isolates and four MDR isolates showed an AMO/CLAV + EMB positive interaction. EMB has an action on intracellular and extracellular bacilli and was introduced in antituberculosis therapy with the objective of combating drug resistance.[3] However, EMB is associated with dose-dependent ocular toxicity and causes permanent vision damage or other side effects. Thus, a reduction of the EMB dosage may help reduce side effects and even reduce the treatment time. A lowering of the MIC of EMB through a combination with AMO/CLAV may be crucially important for increased activity at the site of TB infection, which is difficult by the drug to achieve effective concentrations at this site.[6]

Abate and Miorner[2] tested the AMO/CLAV + EMB combination using the BACTEC method and also obtained encouraging results against *M. tuberculosis* isolates as we obtained with REDCA. They observed ≥ four times reduction of MIC values in 29 of the 30 isolates.

An additional advantage of using the old and classical antituberculosis first-line drugs combined with AMO/CLAV, in cases of resistant TB, is that all of the drugs in this regimen are orally administered, which differs from other new β-lactam antibiotics that have shown therapeutic actions against *M. tuberculosis*. Oral administration is associated with better patient compliance and is a better alternative for pediatric patients.

Clear differences have been reported in the activity of AMO/CLAV alone and combined with antituberculosis drugs against *M. tuberculosis* in some studies. The benefit of the addition of AMO/CLAV to antituberculosis drug-containing regimens should be explored, since *in vitro* results in this study indicated synergism. These combinations may be a promising alternative for the treatment of resistant TB or even in reducing the dosage of the drugs, to reduce the side effects.

## Conflicts of interest

The authors have none to declare.

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