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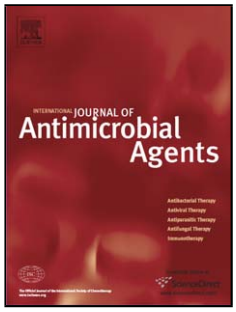

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The role of efflux pumps in macrolide resistance in *Mycobacterium avium* complex

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ABSTRACT

Mycobacterium avium complex (MAC) is clinically important since it can cause severe infections in acquired immune deficiency syndrome (AIDS) patients and other immunocompromised individuals. Use of the macrolides clarithromycin and azithromycin has improved the outcome of MAC infections, but therapeutic failure is still a major problem. In this work, we studied efflux pump activity in MAC clinical strains and evaluated the contribution of active efflux to macrolide resistance. Eighteen clinical strains isolated from AIDS patients were evaluated for macrolide resistance in the presence and absence of the efflux pump inhibitors (EPIs) thioridazine, chlorpromazine and verapamil. The efflux activity of these strains was then assessed by a semi-automated fluorometric method that detects extrusion of ethidium bromide (EtBr), a known efflux pump substrate. Resistance to clarithromycin was significantly reduced in the presence of thioridazine, chlorpromazine and verapamil. The same EPIs were effective in decreasing the efflux of EtBr from MAC cells. Moreover, increased retention of [^{14}C]-erythromycin in the presence of these EPIs further demonstrated that active efflux contributes to MAC resistance to macrolides. This study demonstrates that efflux pumps play an important role in MAC resistance to antibiotics.

1. Introduction

Mycobacterium avium complex (MAC) comprises a heterogeneous group of environmental mycobacteria whose clinical relevance has acquired greater significance in recent decades because it causes severe infections in acquired immune deficiency syndrome (AIDS) patients and other immunocompromised individuals [1–4]. The classic definition of MAC includes the genetically distinct species *Mycobacterium avium* and *Mycobacterium intracellulare* [5]. *Mycobacterium avium* is more prevalent in clinical and environmental samples and is mainly responsible for disseminated MAC disease in human immunodeficiency virus (HIV) patients, whereas *M. intracellulare* is an important contributor to MAC-associated pulmonary infections in immunocompetent or non-HIV patients [2,6–8].

MAC infections are difficult to treat owing to the intrinsic multidrug resistance of the organism, which is assumed to be a consequence of decreased permeability of the cell wall, mainly due to the composition of the thick arabinogalactan–peptidoglycan–mycolic acid layer that is present in all mycobacteria. However, exclusively in MAC strains, this layer is surrounded by a second electron-dense layer of serovar-specific glycopeptidolipids [5]. Thus, the wide range of resistance shown by MAC isolates to most antimicrobial drugs has been ascribed to the composition of its unique cell wall, the integrity of which, when affected, results in increased susceptibility to a variety of drugs [2,3,9,10]. The only antimicrobial agents for which a correlation has been demonstrated between in vitro susceptibility results and clinical outcome in controlled clinical trials are the macrolides clarithromycin and azithromycin.

Because susceptibility to clarithromycin predicts susceptibility to azithromycin, the Clinical and Laboratory Standards Institute (CLSI) guidelines [11] state that clarithromycin should be the only drug routinely evaluated for activity against MAC. There are no CLSI interpretive guidelines for other drugs since in vitro results are not predictive of clinical outcome and may be misleading [11]. Nevertheless, other drugs such as rifabutin, ethambutol, amikacin, clofazimine, linezolid and the fluoroquinolones are relatively effective in controlling MAC bacteraemia as long as they are able to reach their internal target and the target has not mutated [3,10].

It is now known that efflux pumps contribute to intrinsic resistance of mycobacteria to antibiotics. Moreover, when these efflux pumps are overexpressed they extrude a wide variety of chemically and structurally unrelated noxious compounds from the cell, thereby preventing them from reaching their intended targets [12–16]. Although overexpressed activity of efflux pumps may account for multidrug-resistant (MDR) phenotypes of some MAC and *Mycobacterium tuberculosis* clinical strains, evidence in support of this possibility is essentially absent.

The activity of efflux pumps in *M. tuberculosis* responsible for induced high-level resistance to isoniazid can be inhibited by efflux pump inhibitors (EPIs) such as reserpine [12,13,17]. On the other hand, intrinsic efflux activity of MAC strains has been shown to be inhibited by the phenothiazines thioridazine and chlorpromazine as well as by verapamil [18,19].

We have previously demonstrated the presence of an intrinsic efflux system in *M. avium* ATCC 25291^T with the aid of a semi-automated fluorometric method [19]. The study described here uses the same methodology to detect efflux pump activity in MAC clinical strains and correlates this activity with a macrolide-resistant phenotype.

2. Materials and methods

2.1. Materials

Middlebrook 7H9 broth and oleic acid–albumin–dextrose–catalase (OADC) supplement were purchased from Difco (Detroit, MI). Ethidium bromide (EtBr), glucose, phosphate-buffered solution (PBS), potassium phosphate, lithium chloride, chlorpromazine, thioridazine, verapamil and erythromycin were purchased from Sigma-Aldrich Química SA (Madrid, Spain). Clarithromycin was obtained from Abbott Laboratories (Abbott Park, IL). [N-Methyl-^{14}C]-erythromycin and the liquid scintillation cocktail ULTIMA GOLD F were purchased from PerkinElmer (Waltham, MA). All solutions were prepared on the day of the experiment.

2.2. Bacteria

The following MAC strains were used in this study: the reference strains *M. avium* subsp. *avium* ATCC 25291^T and *M. intracellulare* ATCC 13950^T; *M. avium* 104, a common MAC representative whose genome is sequenced; and 17 clinical strains isolated from respiratory specimens from AIDS patients, received in our laboratory from hospitals of the Greater Lisbon area for routine

mycobacterial isolation and identification. All specimens were processed by the conventional mycobacteriological NaOH-NALC method [20] and aliquots were collected for acid-fast staining (Ziehl–Neelsen) and inoculation of MGIT tubes of the BACTEC™ MGIT™ 960 system (Becton-Dickinson Diagnostic Instrument Systems, Towson, MD). MAC strains present in full-grown cultures were identified as *M. avium* or *M. intracellulare* by the AccuProbe® system (Gen-Probe Inc., San Diego, CA) according to the manufacturer's instructions. For the following studies the strains were grown at 37 °C in Middlebrook 7H9 broth or Middlebrook 7H11 solid media, both supplemented with 10% OADC.

2.3. Determination of minimum inhibitory concentrations (MICs)

MICs for the EPIs thioridazine, chlorpromazine and verapamil and for the antibiotics clarithromycin and erythromycin, alone and in the presence of an EPI, were determined by the broth microdilution method according to CLSI guidelines [11]. Briefly, MAC strains were grown in 7H9/OADC medium at 37 °C to an optical density at 600 nm (OD_{600}) of 0.8. Bacterial cultures were diluted with PBS to McFarland No. 0.5 standard and the inoculum prepared with an aliquot of the suspension was diluted to 1:100. Aliquots of 0.1 mL of the inoculum were transferred to wells of a 96-well plate containing 0.1 mL of 7H9/OADC medium with two-fold serial dilutions of each agent. The inoculated plates were incubated at 37 °C until growth in the agent-free control well was evident (5–7 days). The MIC was defined as the lowest concentration of compound that inhibited visible growth.

2.4. Ethidium bromide efflux assay by a semi-automated fluorometric method

EtBr extrusion from MAC cells was assessed by a semi-automated fluorometric method as described previously [19,21]. Briefly, mycobacterial cells were grown in 7H9/OADC medium at 37 °C until an OD_{600} of 0.8. The culture was centrifuged at 13 000 rpm for 3 min, the supernatant was discarded and the pellet was washed once and re-suspended in PBS. After adjusting the OD_{600} to 0.4, mycobacteria were exposed to conditions that promoted maximum accumulation of EtBr: EtBr at 0.5× MIC for each strain; no glucose; presence of verapamil at 0.5× MIC; and incubation at 25 °C for 60 min. EtBr-loaded cells were centrifuged at 13 000 rpm for 3 min and re-suspended in EtBr-free PBS. After adjusting the OD_{600} to 0.4, glucose was added at a final concentration of 0.4%, aliquots of 0.095 mL were transferred to 0.2 mL microtubes and EPIs were added. Fluorescence was measured in a real-time Rotor-Gene™ 3000 thermocycler (Corbett Research, Sydney, Australia) using 530 nm band-pass and a 585 nm high-pass filters as the excitation and detection wavelengths, respectively. Fluorescence data were acquired every 60 s for 30 min at 37 °C. Efflux activity was quantified by comparing the fluorescence data obtained for mycobacteria under conditions that allow maximum efflux (incubation at 37 °C, in the presence of glucose and absence of EPI) against the data from the control tube that contains the EtBr-loaded cells under conditions that inhibit efflux (presence of EPI and no glucose). Thus, the relative fluorescence corresponds to the ratio of fluorescence that remains per unit of time relative to the EtBr-loaded cells [19,21]. Each experiment was conducted at least three times and the results obtained did not vary qualitatively.

2.5. [^{14}C]-Erythromycin accumulation assay

MAC cultures were grown in 7H9/OADC at 37 °C until an OD_{600} of 0.8. Cells were harvested by centrifugation at 13 000 rpm for 3 min, washed once with PBS and the pellet was re-suspended in the same buffer. The OD_{600} was adjusted to 0.4 with PBS containing glucose at a final concentration of 0.4% and 10 μM of [^{14}C]-erythromycin (specific activity 50 mCi/mmol) was added. The bacterial suspension containing radiolabelled erythromycin was split into two replicate sets: one set of triplicate tubes received 0.05 mL of an EPI at 0.5× MIC, whilst the other set of triplicate tubes received an equal volume of PBS. Following incubation at 37 °C for 16 h, 1.0 mL aliquots were filtered on Whatman GF/C filters. The filters were washed twice with 3.0 mL of cold 100 mM lithium chloride–50 mM potassium phosphate buffer (pH 7.0), dried overnight at 37 °C and transferred to scintillation vials containing the liquid scintillation cocktail ULTIMA GOLD F. The counts per minute (CPM) of each triplicate tube of the replicate sets corresponding to [^{14}C]-erythromycin that remained inside the cells were obtained with the aid of a Beckman LS6500 scintillation counter (Beckman Coulter, Fullerton, CA). The data presented are the average CPM per replicate set of three tubes.

2.6. Screening for mutations in the 23S rRNA gene associated with macrolide resistance

Mycobacterial genomic DNA was extracted using a QIAamp DNA Mini Kit (QIAGEN GmbH, Hilden, Germany) according to the manufacturer's instructions. A 420-bp DNA fragment, spanning positions 1886–2305 of domain

V of the 23S rRNA gene, was amplified by polymerase chain reaction (PCR) using primers 23S_FI (TTTAAGCCCCAGTAAACGGC) and 23S_RIII (GTCCAGGTTGAGGGAACCTT) as described previously [22]. The reaction mixture (50 μL) contained 2.5 U of *Taq* polymerase (Fermentas Inc., Ontario, Canada), 1× *Taq* buffer (Fermentas Inc.), 20 pmol of each primer, 200 mM dNTP and 1.75 mM $MgCl_2$. PCR was conducted in a Mastercycler personal 5332 thermocycler (Eppendorf AG, Hamburg, Germany) and amplification conditions were as follows: DNA denaturation at 94 °C for 4 min; 35 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min and extension at 72 °C for 1 min; and a final extension at 72 °C for 10 min. Amplification products were visualised in 1% agarose gels, purified and sequenced in both strands. Sequences were analysed and aligned using the programs BioEdit version 7.0.9, and ClustalW, respectively, with reference to the 23S rRNA gene of the *M. avium* 104 genome (GenBank accession no. CP000479.1).

3. Results

The MICs for clarithromycin and erythromycin against the identified strains are summarised in Table 1. Among the clinical strains tested, *M. avium* 47/07, *M. avium* 386/08, *M. intracellulare* 253/07 and *M. intracellulare* AL2 presented clinically significant resistance to clarithromycin [breakpoints according to CLSI guidelines: susceptible, ≤ 16 mg/L; intermediate, 32 mg/L; and resistant, ≥ 64 mg/L] and a high MIC for erythromycin. This high-level clarithromycin/erythromycin resistance is invariably associated with a mutation

in adenine 2058 or 2059 in domain V of the 23S rRNA gene and is the main mechanism of resistance accounting for this phenotype [23–25].

To determine whether the drug susceptibility phenotype of these isolates was affected by the presence of EPIs, the MIC for each antibiotic was determined in the absence and presence of 0.5× MICs of thioridazine, chlorpromazine and verapamil (Table 2). It is important to note that 0.5× MIC of each of the agents listed has no effect on the viability of the organisms (data not shown). This is a condition that must be satisfied if the effect of the EPI on the MIC of an antibiotic is to be exclusively that against the efflux system itself [19,21]. As shown by the results presented in Table 1, the EPIs chlorpromazine and thioridazine reduced the MIC of clarithromycin for 12 of the 20 isolates tested. The effects of these EPIs on the MICs of erythromycin were also evident for seven strains. Verapamil promoted a significant reduction in the MICs for clarithromycin and erythromycin in four and eight strains, respectively. These results suggest that MAC strains have one or more chlorpromazine- and thioridazine-sensitive efflux pump(s) that recognise clarithromycin as a substrate. However, in the particular case of the strains with high-level resistance to clarithromycin, the reduction in the MICs promoted by the EPIs does not reach a level of susceptibility, i.e. MIC ≤ 16 mg/L.

Screening for mutations in domain V of the 23S rRNA gene revealed the presence of point mutations in position 2058 in three strains that presented high-level resistance to clarithromycin (*M. avium* 47/07, A→G; *M. intracellulare* 253/07, A→C; and *M. intracellulare* AL2, A→C). Substitution of the adenine at

position 2058 by guanine or cytosine has been described in association with high-level resistance to clarithromycin [22,23,25]. The other isolate showing phenotypic resistance to clarithromycin (*M. avium* 386/08), albeit at a lower level, presented a wild-type sequence in this region of the 23S rRNA gene. This phenotype could result from mutation(s) located outside this region or could be due to a different resistance mechanism(s), including efflux.

The efflux activity of MAC reference and clinical strains was assessed by a semi-automated fluorometric method in the presence and absence of the EPIs. As shown in Fig. 1, efflux of EtBr was detected both in reference and clinical strains (represented by *M. avium* 386/08 and *M. intracellulare* AL2) at 37 °C in the presence of glucose. Moreover, this efflux activity decreased in the presence of verapamil and thioridazine (Fig. 1). A similar effect was observed with chlorpromazine (data not shown). Whilst both *M. avium* strains showed similar EtBr efflux activity, for *M. intracellulare* efflux was more evident in the clinical strain whilst only a basal efflux activity was registered for *M. intracellulare* ATCC 13950^{T}. These results provide further evidence that efflux activity is involved in resistance to macrolides in both *M. avium* and *M. intracellulare* clinical strains. Evidence for such activity was also provided by studying the retention of radiolabelled erythromycin in order to compare the intracellular accumulation of this antibiotic in the presence or absence of an EPI. As shown in Fig. 2, the presence of thioridazine and verapamil promotes intracellular retention of [^{14}C]-erythromycin, in accordance with the results obtained for erythromycin MIC determination in the presence and absence of these EPIs (Table 1). These results confirm the previous assumption that at

least one active efflux system is involved in the extrusion of macrolides and contributes to the resistance of MAC to these drugs.

4. Discussion

Mycobacteria belonging to MAC are still the most frequent cause of opportunistic bacterial infection in patients with AIDS. Treatment of MAC disease remains difficult and requires months of multiple therapy, although it has been greatly improved with the introduction of the extended-spectrum macrolides clarithromycin and azithromycin, which are far more effective than other antimicrobial agents against MAC infections [1–4]. However, cross-resistance between clarithromycin and azithromycin has been detected and, although monotherapy with clarithromycin (or azithromycin) significantly reduced levels of MAC bacteraemia, it is usually followed by relapses from macrolide-resistant strains. Consequently, other antimycobacterial drugs must be used in combination with clarithromycin to prevent the emergence of macrolide resistance [26].

Efflux of antibiotics has been identified as a relevant contributor to bacterial resistance in clinical practice and is now recognised as an important cause of intrinsic antibiotic resistance in mycobacteria [12,18,19]. In this work, we investigated the contribution of active efflux to macrolide resistance in clinical MAC strains isolated from AIDS patients. In these strains, resistance to clarithromycin was significantly reduced in the presence of EPIs such as the calcium-channel inhibitors thioridazine and chlorpromazine as well as the calcium ion influx inhibitor verapamil, which have been shown to inhibit efflux

activity in *M. avium* ATCC 25291^T [19]. In this work, we have also shown that these EPIs effectively reduce the MIC of clarithromycin for most clinical strains tested. The same EPIs were effective in decreasing efflux from MAC cells loaded with EtBr, a known efflux pump substrate. These efflux assays highlighted the efflux activity in the four high-level resistant MAC strains identified in this study (two *M. avium* and two *M. intracellulare* strains), demonstrating that besides the presence of mutations in the 23S rRNA gene in three of these strains, this high-level resistance also results from efflux activity, as already suggested by the reduction of MICs for clarithromycin and erythromycin in the presence of the EPIs (Table 1). Retention of [^{14}C]-erythromycin by the same inhibitors further demonstrated that active efflux contributes to MAC resistance to macrolides.

Prolonged exposure to antimycobacterial compounds, such as in MAC therapy, may render the mycobacterial population increasingly resistant by means of increased efflux activity, from which mutants emerge with an increased probability, explaining the relapses from macrolide-resistant strains in patients subjected to monotherapy, especially in AIDS patients where the immune system is weakened; there is thus the need to add other mycobacterial drugs [10,26]. Therefore, agents that inhibit mycobacterial efflux pumps not only render the organism more susceptible to an antibiotic but also reduce the probability of selection of spontaneously arising mutants and may become important antimycobacterial therapy adjuvants and a source of new antimycobacterial compounds.

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Fig. 1. Efflux of ethidium bromide (EtBr) by *Mycobacterium avium* ATCC 25291^{T}, *Mycobacterium intracellulare* ATCC 13950^{T} and the clinical strains *M. avium* 386/08 and *M. intracellulare* AL2. Assays were conducted at 37 °C, with or without an efflux pump inhibitor (EPI). Relative fluorescence was obtained by normalisation of data against the conditions of no efflux (presence of EPI and no glucose). Efflux of EtBr was inhibited by verapamil (VP) and thioridazine (TZ) used at 0.5× the minimum inhibitory concentration.

Fig. 2. Retention of [^{14}C]-erythromycin (Ery) by *Mycobacterium avium* complex reference and clinical strains. Intracellular accumulation of [^{14}C]-Ery was determined in the presence and absence of verapamil (VP) or thioridazine (TZ) at 0.5× the minimum inhibitory concentration. Values [expressed as counts per minute (CPM)] are the average of three replicate tubes. Error bars indicate standard deviations.

Table 1

Effect of the efflux pump inhibitors (EPIs) thioridazine (TDZ), chlorpromazine (CPZ) and verapamil (VER) on the minimum inhibitory concentrations (MICs) of clarithromycin and erythromycin against *Mycobacterium avium* complex strains [a]

Strain	MIC (mg/L) [b]							
	Clarithromycin				Erythromycin			
	No EPI	TDZ	CPZ	VER	No EPI	TDZ	CPZ	VER
M. avium ATCC 25291^T	4	2	2	2	16	**0.5**	**0.5**	**4**
M. avium 104	8	8	8	8	256	256	256	256
M. avium HSB1	8	**2**	**2**	4	256	256	256	256
M. avium HSB2	8	**2**	**2**	8	256	256	256	256
M. avium HSB3	8	**2**	**2**	8	256	**64**	**16**	128
M. avium HSB4	8	**1**	**1**	8	256	128	128	256
M. avium HSB5	8	**2**	**2**	**2**	256	256	256	256
M. avium HSB6	8	**2**	**2**	8	256	256	256	256
M. avium AL1	8	**2**	**1**	**1**	128	64	**16**	**16**
M. avium 47/07	1024	1024	1024	512	512	256	512	**128**
M. avium 386/08	512	512	**128**	512	1024	**256**	**256**	512
M. intracellulare ATCC 13950^T	0.25	0.25	0.25	0.25	0.06	0.06	0.06	0.06
M. intracellulare 101/07	8	8	4	8	256	256	256	**64**
M. intracellulare 278/07	8	8	8	8	256	**16**	**16**	256
M. intracellulare 308/07	1	0.5	0.5	1	256	256	256	**16**
M. intracellulare 421/07	1	0.5	0.5	1	256	256	256	256
M. intracellulare 30/08	1	**0.25**	0.5	**0.25**	256	256	256	**64**
M. intracellulare 92/08	2	**0.5**	1	**0.125**	256	256	128	256
M. intracellulare 253/07	1024	1024	**256**	1024	2048	1094	**512**	**256**
M. intracellulare AL2	2048	**256**	2048	1024	2048	**256**	1094	**256**

[a] EPIs were used at 0.5× MIC. A four-fold reduction was considered to denote a significant synergistic effect between the antibiotic and the EPI and is identified in bold.

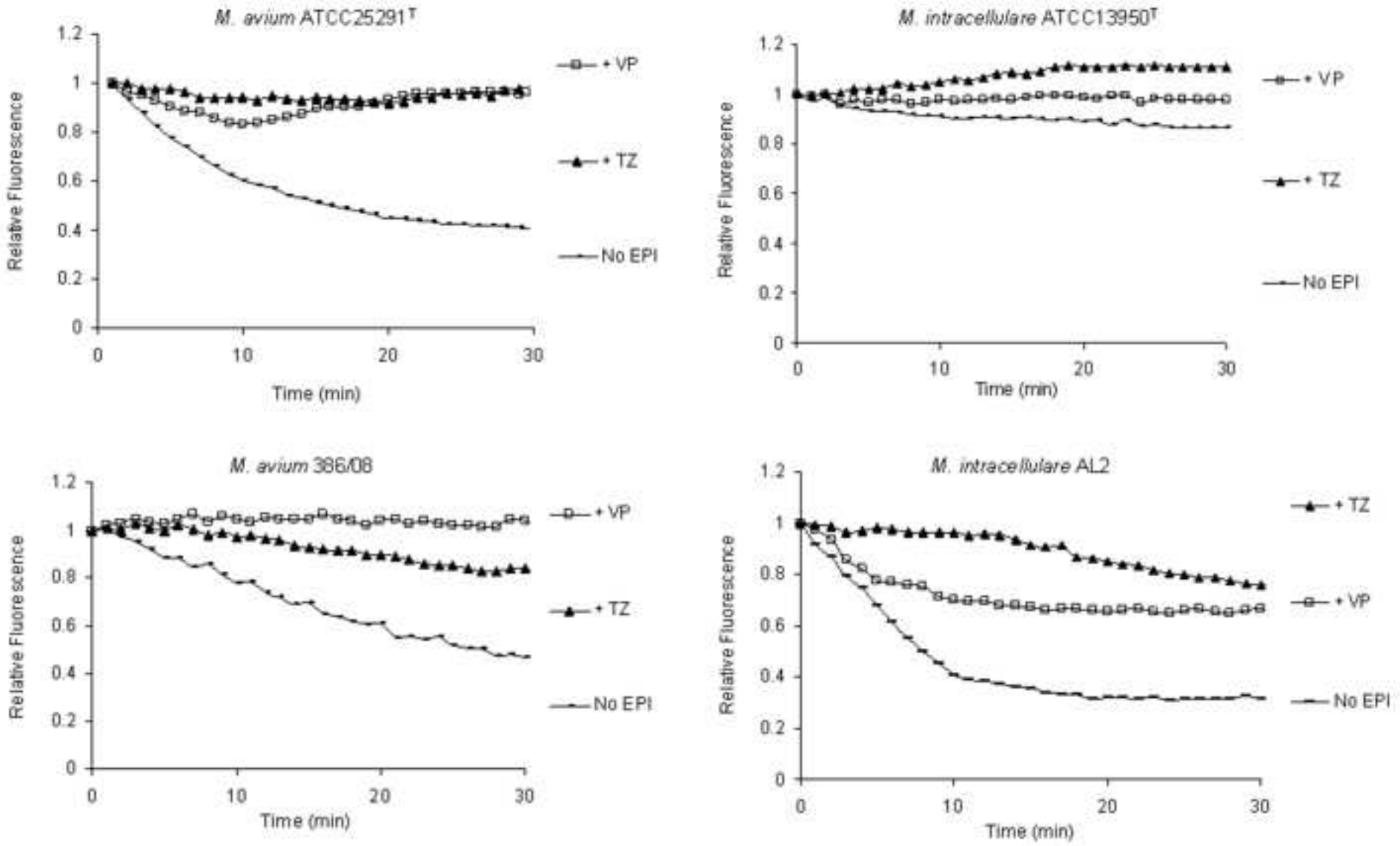
[b] Breakpoints for clarithromycin MIC determination by microdilution in Middlebrook 7H9 broth (pH 6.8) were as follows: susceptible, ≤ 16 mg/L; intermediate, 32 mg/L; resistant, ≥ 64 mg/L [11].

Table 2

Minimum inhibitory concentrations (MICs) of ethidium bromide (EtBr) and the efflux pump inhibitors thioridazine (TDZ), chlorpromazine (CPZ) and verapamil (VER) determined for *Mycobacterium avium* complex clinical and reference strains

Strain	MIC (mg/L)			
	TDZ	CPZ	VER	EtBr
M. avium ATCC 25291^T	20	25	800	6.25
M. avium 104	25	25	500	6.25
M. avium HSB1	25	25	1000	16
M. avium HSB2	25	25	500	8
M. avium HSB3	25	25	1000	16
M. avium HSB4	25	25	1000	16
M. avium HSB5	6.25	25	1000	8
M. avium HSB6	25	25	500	8
M. avium AL1	10	25	500	6.25
M. avium 47/07	12.5	12.5	500	12.5
M. avium 386/08	25	12.5	1000	12.5
M. intracellulare ATCC 13950^T	12.5	50	1000	2
M. intracellulare 101/07	12.5	12.5	500	12.5
M. intracellulare 278/07	12.5	12.5	500	6.25
M. intracellulare 308/07	12.5	12.5	500	12.5
M. intracellulare 421/07	12.5	12.5	500	12.5
M. intracellulare 30/08	12.5	12.5	500	12.5
M. intracellulare 92/08	12.5	12.5	500	12.5
M. intracellulare 253/07	6.25	12.5	500	4
M. intracellulare AL2	6.25	12.5	500	4

Edited Figure 1



Edited Figure 2