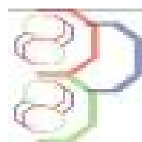

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High *Rv1819c* efflux pump gene expression in persistent *Mycobacterium tuberculosis* clinical isolates.

Sungai Mazando[1], Prof. Clemence Zimudzi[1] , Moses Zimba[1], Prof Alex Pym[2] and Prof Peter Mason[3]
[1] Biological Sciences Department, University of Zimbabwe, 630 Churchill Avenue, Mount Pleasant Harare, Zimbabwe. +263- 4-303211
[2] KwaZulu-Natal Research Institute for Tuberculosis and HIV (K-RITH), K-RITH Tower Building, level 3, Nelson R. Mandela School of Medicine, 719 Umbilo Road, 4001 Durban, South Africa. +27 31 260 4991
[3] Biomedical Research and Training Institute, No. 10 Seagrave road, Avondale, Harare, Zimbabwe. +263-4-333091
Corresponding Author, Sungai Mazando, Biological Sciences Department, University of Zimbabwe, 630 Churchill Avenue, Mount Pleasant Harare, Zimbabwe.
Phone: +263- 773-425250 Fax: +263-4-308941 sungimazando@gmail.com

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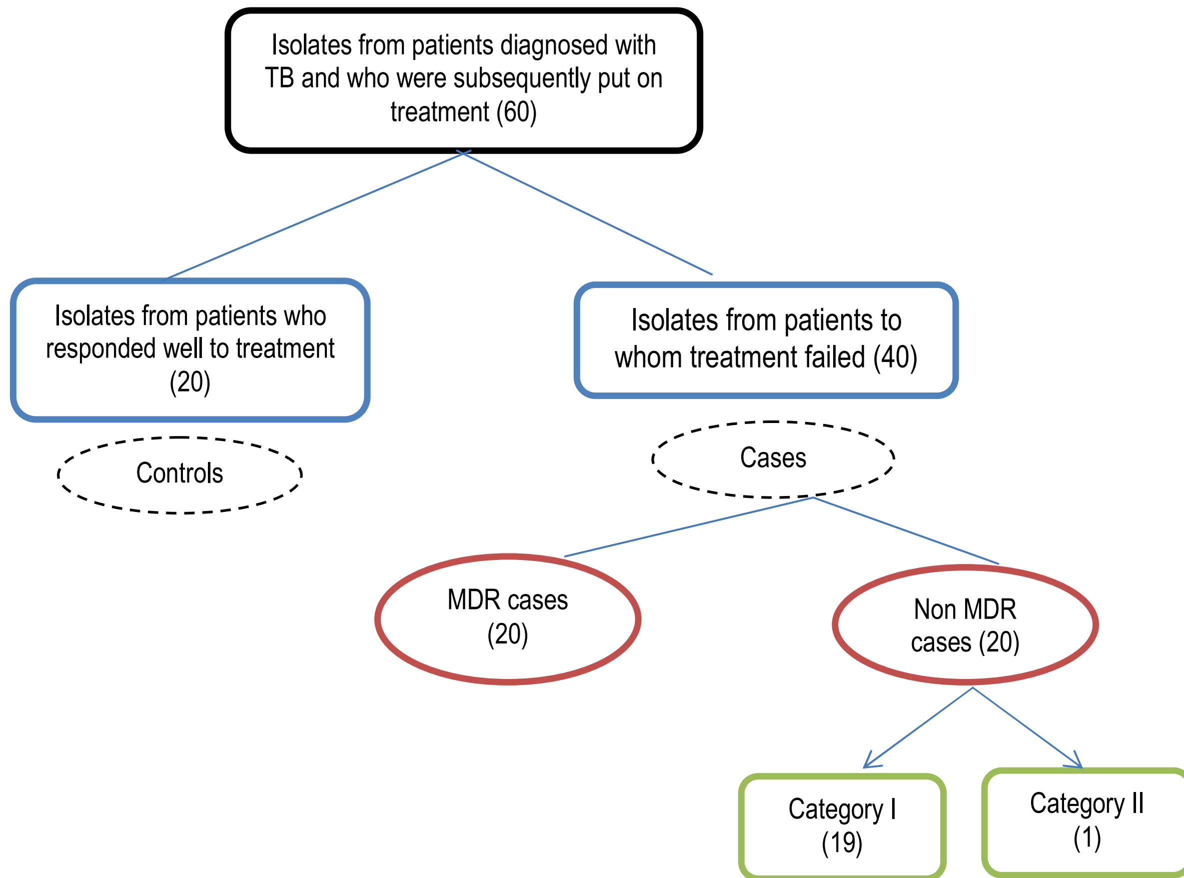
ABSTRACT
Objectives: Interest in mycobacterial persistence is rising, stemming from the current TB drugs being inadequate at killing dormant or persistent tubercle bacilli resulting in a lengthy chemotherapy. This study hypothesized that efflux pumps could be a risk factor to TB persistence, such that TB bacilli are not rapidly cleared during the intensive phase of treatment.
Methodology and Results: M. tuberculosis isolates from patients whose sputum smear had remained positive despite being subjected to two months intensive phase of TB treatment were employed as cases in this study. Isolates from patients who successfully seroconverted to negative sputum smear were the controls. An investigation was done on whether *Rv1819c* efflux pump gene expression and its correlation with the cell's ability to efflux ethidium bromide (a common efflux substrate) is associated with clinical persistence of TB bacilli. Efflux pump gene expression differed significantly between the treatment failures and treatment successes according to the Mann-Whitney Test at $p \leq 0.05$. Efflux of ethidium bromide by *Mycobacterium tuberculosis* isolates revealed that isolates from treatment failures rapidly efflux ethidium bromide more than isolates from treatment successes or the H37Rv control strains.
Conclusions and application of findings: High *Rv1819c* gene expression at baseline can be associated with tuberculosis treatment failure even when the *M. tuberculosis* does not have established resistance causing mutations. Logistic regression analysis of the association between efflux pump gene expressions revealed that increasing *Rv1819c* expression also increases the risk of treatment failure. The results of this study informs future research and clinical practice of the importance of evaluating efflux pump inhibitor drug candidates in combination with traditional anti-TB drugs in the quest to shorten TB treatment duration as well as improving TB treatment outcomes.

INTRODUCTION

During TB chemotherapy, sputum bacillary counts decrease in a characteristic biphasic manner. For example, with isoniazid, greater than 99 % of the initial sputum bacillary load is killed during the first 2 days of treatment, after which the rate of killing drops off markedly (Szumowski *et al*., 2013) The residual bacteria are a phenotypically resistant, ''drug tolerant'' population. TB drug minimum inhibitory concentrations remain unchanged in the drug tolerant/persistent population. Empirical studies have shown that it takes months of therapy to eradicate these bacteria and produce a stable cure (Mitchison and Davies, 2012). Interest in mycobacterial persistence and dormancy stems primarily from the frustration that the current TB drugs have proven inadequate at killing dormant or persistent tubercle bacilli resulting in a lengthy chemotherapy, which leads to poor compliance amongst patients ultimately increasing the risk of the development of drug resistance (Zhang *et al*., 2012). The classical definition of persistence refers to a phenomenon where otherwise drug-susceptible microorganisms exhibit the ability to survive indefinitely within mammalian tissues despite continued exposure to the correct antimicrobial drug or drugs (Zhang, 2004). Persister formation has been reported to be promoted by epigenetic factors in either a deterministic or a stochastic manner (Dorr *et al*., 2009). Persister cells may exhibit morphologies that are not distinguishable from those of susceptible cells (such as the ones used in this study) or may be distinct in some way (Zhang *et al*., 2004). The yin-yang model of microbial persistence depicts a dynamic bacterial population consisting of non growing and growing subpopulations in various metabolic states in a continuum. The persister subpopulation is depicted as heterogeneous, consisting of a continuum of diverse subpopulation of bacilli because of induced expression of persister genes or stochastic events (Sarathy *et al*., 2013). The yin-yang model is used to try to explain why, after a 2-month intensive-phase treatment with rifampin (RIF), isoniazid (INH), ethambutol (EMB) and pyrazinamide (PZA), the remaining persister bacilli can still revert to a growing form, which can still be killed by RIF and INH in the subsequent 4-month continuation phase of treatment. Persisters have a phenotypic resistance or tolerance to antibiotics that is noninheritable and their progeny remain fully susceptible to antibiotics upon regrowth (Zhang *et al*., 2012).This phenotypic drug resistance, also known as drug tolerance, has been previously attributed to slowed bacterial growth *in vivo*. Recent findings challenge this model and instead implicate macrophage-induced mycobacterial efflux pumps in antimicrobial tolerance and persistence (Szumowski *et al*., 2013). Although mycobacterial efflux pumps may have originally served to protect against environmental toxins, in the pathogenic mycobacteria, they appear to have been repurposed for intracellular growth and virulence. The increased expression of efflux systems significantly lowers the intracellular concentration of many antibiotics thus reducing their clinical efficacy (Singh *et al*., 2011). Analysis of gene expression of efflux pumps associated with multidrug resistance in *M. tuberculosis* and its correlation with the cell's ability to efflux ethidium bromide (a common efflux substrate), provides strong evidence whether clinically persistent TB strains demonstrate increased efflux activity and expression compared to susceptible strains. This study demonstrates that the drug tolerance can be reverted by efflux inhibitors, in support of their potential role as adjuvants in anti-tuberculosis therapy and prevention of clinical persistence.

MATERIALS AND METHODS

Ethics statement: This study was approved by the ethics committee of the Biomedical Research and Training Institute of Zimbabwe. All patients involved in the study provided written informed consent.



**Treatment failures are defined as patients who were still sputum positive after 2 months of chemotherapy*
Figure 1: Study design

***M. tuberculosis* isolates:** Sixty frozen mother cultures of TB isolates, which could be matched to patients with a known treatment history, were used in this study (Figure 1). These samples were obtained from a Mycobacterial sample Repository at the Biomedical Research and Training Institute National reference laboratory deposited from July 2004 to January 2008. The isolates were selected from patients who had presented with clinical symptoms of TB and had not received TB treatment before. Equal numbers of males and females were selected. Clinical data of the patients diagnosed with drug susceptible TB and MDR TB were stripped of all patient identifiers and assigned anonymous study Identifications(Ids). Data on new cases/previously treated cases, age, sex, HIV status and TB treatment history was collected using a coded data collection form and captured using an electronic database (Epi-Info ™). The study employed a retrospective case study recruiting isolates from patients who had not responded to normal DOTS treatment as evidenced by a positive sputum sample after two months of treatment as cases as outlined in Figure 1. Of the 60 isolates, 40 isolates were cases and 20 isolates from patients who had responded to treatment within the first two months of treatment served as controls. Of the 40 cases, 20 were genetically susceptible but phenotypically tolerant while 20 were multi drug resistant as confirmed

by GeneXpert MTB/RIF test, GenoType MTBDR*plus* test and by Lowenstein-Jensen (L-J) culture method. Before being subjected to tests, sputum samples were first digested and decontaminated by the Kubica N-acetyl-L-cysteine NaOH method (Ratnam *et al*., 1987) After decontamination, the concentrated sediment was suspended in sterile phosphate buffer (pH 7.0) followed by auramine-rhodamine acid-fast staining. Specimens positive by fluorochrome staining were further confirmed by Ziehl-Neelsen staining. An aliquot of the decontaminated specimens was cultured on Lowenstein-Jensen solid medium while the remaining decontaminated specimen was stored at -20°C. Isolates of the *M. tuberculosis* complex were detected in all samples included in this study.

Antimicrobial agents: Rifampicin (RIF), isoniazid (INH), ethambutol (EMB), ciprofloxacin (CIP), levofloxacin (LXF) and kanamycin (KM) for MIC determination and efflux inhibitors carbonylcyanide-3-chlorophenylhydrazone (CCCP), verapamil and thioridazine, as well as the efflux substrate ethidium bromide (EtBr), were purchased from Sigma-Aldrich (Steinheim, Germany).

Assay of ethidium bromide accumulation and efflux in intact cells: The detection of ethidium bromide accumulation and efflux on a real-time basis by the *M. tuberculosis* isolates was performed using a fluorometric method previously described by Rodriguez *et al*., (2013) with minimum alterations. Ethidium bromide loaded cells were centrifuged at 5,000 g for 5 minutes at room temperature, washed once with 50 mM sodium phosphate buffer (pH 7.2), and resuspended in the same buffer supplemented with 0.4% glucose at an OD_{600} of 0.5. Aliquots of 100 µl of bacterial suspension were transferred into a 96-well plate. Relative fluorescence was acquired every 60 s for 60 min at 37°C in a Synergy HT detection microplate reader (Biotek Instruments), using 495 nm and 580 nm as excitation and detection wavelengths, respectively. To determine the effect of thioridazine, CCCP, and verapamil on the accumulation of ethidium bromide, 10 µl of each compound was added to the corresponding well of the 96-well plate. Each inhibitor was used at ½ the MIC in order not to compromise the cellular viability. Relative fluorescence was acquired every 60 s for 60 min at 37°C in a Synergy HT detection microplate reader (Biotek Instruments), using 495 nm and 580 nm as excitation and detection wavelengths, respectively. In order to allow a comparative analysis of the efflux, the raw data obtained from the fluorimeter was normalized, establishing the ethidium bromide loaded cells as the maximum fluorescence value (relative fluorescence equivalent to 1) that can be obtained during the assay. The relative fluorescence of the tubes used for the measurement of efflux was determined as the ratio between the raw fluorescence data of the efflux and the ethidium bromide loaded cells. The efflux is thus represented as the ratio of fluorescence that remains per unit of time, relatively to the ethidium bromide loaded cells.

Quantification of expression of efflux pump genes by Real Time quantitative PCR (RT-qPCR): All 60 *M. tuberculosis* strains were subcultured in 7H9 medium with OADC supplement plus sub inhibitory concentrations of both INH and RIF at a ¼ of the MIC values. Total bacterial RNA was isolated from mid-exponential-phase cultures at an OD_{600} of 0.8 to 1.0 (50 ml) by the TRIzol® Max™ Bacterial RNA Isolation Kit (Life Technologies, South Africa) according to manufacturer`s instructions. All RNA samples were aliquoted and stored at -20 °C until required. The quality and integrity of the total RNA was assessed using a nanophotometer (Implen, Germany) and agarose gel electrophoresis. After treatment with DNase I (RNase-free) (Life Technologies, South Africa), the lack of DNA contamination of the RNA samples was confirmed by polymerase chain reaction (PCR) amplification of rpoB directly from RNA. The forward and reverse primers are listed in Table 4.

Table 4: Relative expression levels (2-ΔCT value) of six drug efflux genes of clinically persistent and drug sensitive M. tuberculosis isolates

Gene	**Efflux pump family**	**Primer Sequence**	**$2^{-\Delta CT}$ values**					
			Responded to treatment		Did not respond to treatment			
					Non-drug resistant strains		Drug resistant strains	
			Median	25% – 75% value	Median	25% – 75% value	Median	25% – 75% value
Rv1819c	ABC	Forward:5' GCG TCG TAG TTG TTG CGG AAG 3' Reverse:5' TGG ATG GAA TCT GTC GGTGAG C 3'	3.45	3.12 – 4.88	4.48*	2.47 – 7.38	4.28*	2.99 – 6.28

The quantification of the relative mRNA expression level was done using the comparative quantification cycle (Cq) method (Livak and Schmittgen, 2001). Comparison of the relative quantity of the respective mRNA in the H37Rv control strain with that of the isolates was used to determine the relative expression of the efflux pump genes. A single isolate was measured in triplicate using total RNA obtained from three independent cultures of the same isolate. Expression levels identical to that of the H37Rv control strain would have a level of relative expression value equal to 1.

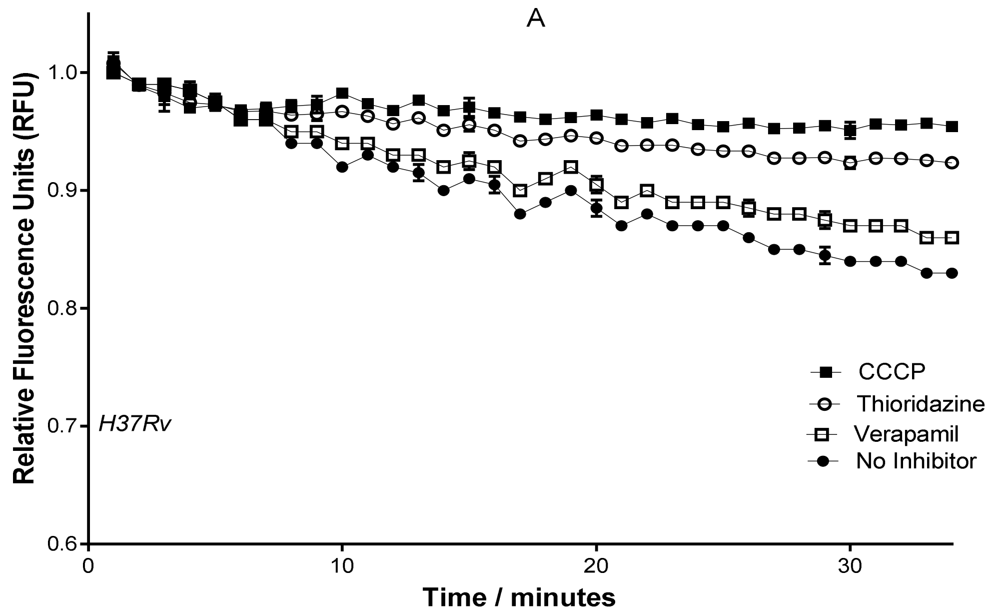
* Efflux gene expression that differs significantly between the treatment failures and treatment successes according to the Mann-Whitney U Test at $p \leq 0.05$.

RT-qPCR assay: The relative expression level of the *Rv1819c* gene that codes for an efflux transporter in *M. tuberculosis* was analyzed by RT-qPCR. The RT-qPCR procedure was performed in a CFX96 Touch™ Real-Time PCR detection system (Biorad) thermocycler and followed the protocol recommended for use with the iTaq™ universal SYBR® Green one-step kit (Biorad).

RESULTS AND DISCUSSION

GenoType MTBDRplus assay: All isolates susceptible or resistant to RIF OR INH were correctly identified as being sensitive or resistant by the MTBDRplus assay, and the results of the MTBDRplus assay were concordant with those obtained with the Lowenstein-Jensen (L-J) culture method and the GeneXpert MTB/RIF test for RIF. Gene mutations conferring RIF resistance on the 20 MDR isolates as identified by the MTBDRplus assay are shown in the Appendix. The mutations in the katG, inhA and rpoB genes identified by the MTBDRplus assay are as shown in the Appendix tables 1 and 2**.**

Assay of ethidium bromide accumulation and efflux in intact cells: The efflux pump substrate EtBr is widely used as a probe in detecting and quantifying efflux activity in bacteria (Rodrigues *et al*., 2011). EtBr fluoresces weakly in aqueous solution when outside cells but becomes strongly fluorescent when concentrated in the cytoplasm of Gram-positive bacteria and in the periplasm of Gram-negative bacteria. High concentrations of ethidium bromide intercalate between nucleic bases of DNA where the binding affinity is high enough to prevent its extrusion from the cell by efflux systems. Efflux of ethidium bromide by *M. tuberculosis* isolates from *clinical persisters* showed greatest activity as reflected by the least concentration of ethidium bromide left in the cell because of efflux activity (Figure 2). The presence of efflux inhibitors decreased the activity of efflux such that less ethidium bromide was effluxed out of the mycobacterial cells (Figure 2). CCCP was the most potent efflux inhibitor followed by thioridazine and to a less extent verapamil, a trend that was also observed in accumulation studies (Rodriguez *et al*., 2013). Multiple efflux inhibitor drugs such as verapamil, reserpine, phenothiazines such as thioridazine, and piperine have been shown to inhibit bacterial efflux pumps *in vitro* (Sharma *et al*., 2010). Macrophage-induced tolerance has been demonstrated to be inhibited by verapamil, a calcium channel antagonist in clinical use for years, which also inhibits multiple bacterial efflux pumps in vitro pumps (Szumowski *et al*., 2013, Adams *et al*., 2014). The activity of most efflux pumps has been shown to be inhibited by several compounds such as verapamil, cccp and thioridazine which have been demonstrated to have efflux pump inhibiting activity against mycobacteria both *in vitro* and *in vitro* (Gupta *et al*., 2013, Amaral *et al*., 2012, Aparna *et al*., 2014).


A
Relative Fluorescence Units (RFU)
Time / minutes
H37Rv
CCCP
Thioridazine
Verapamil
No Inhibitor
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0.9
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20
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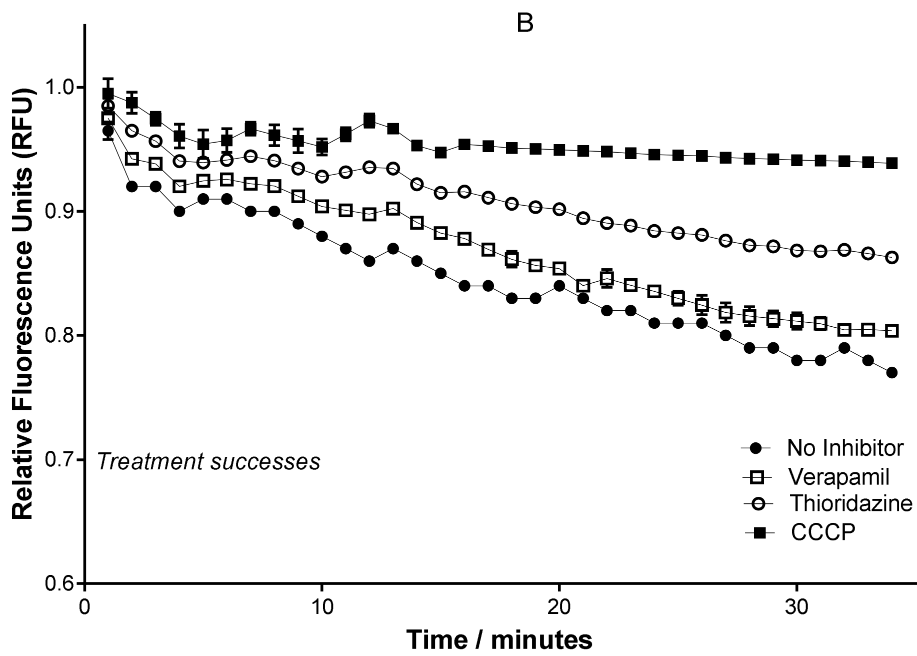
B
Relative Fluorescence Units (RFU)
Time / minutes
Treatment successes
No Inhibitor
Verapamil
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1.0
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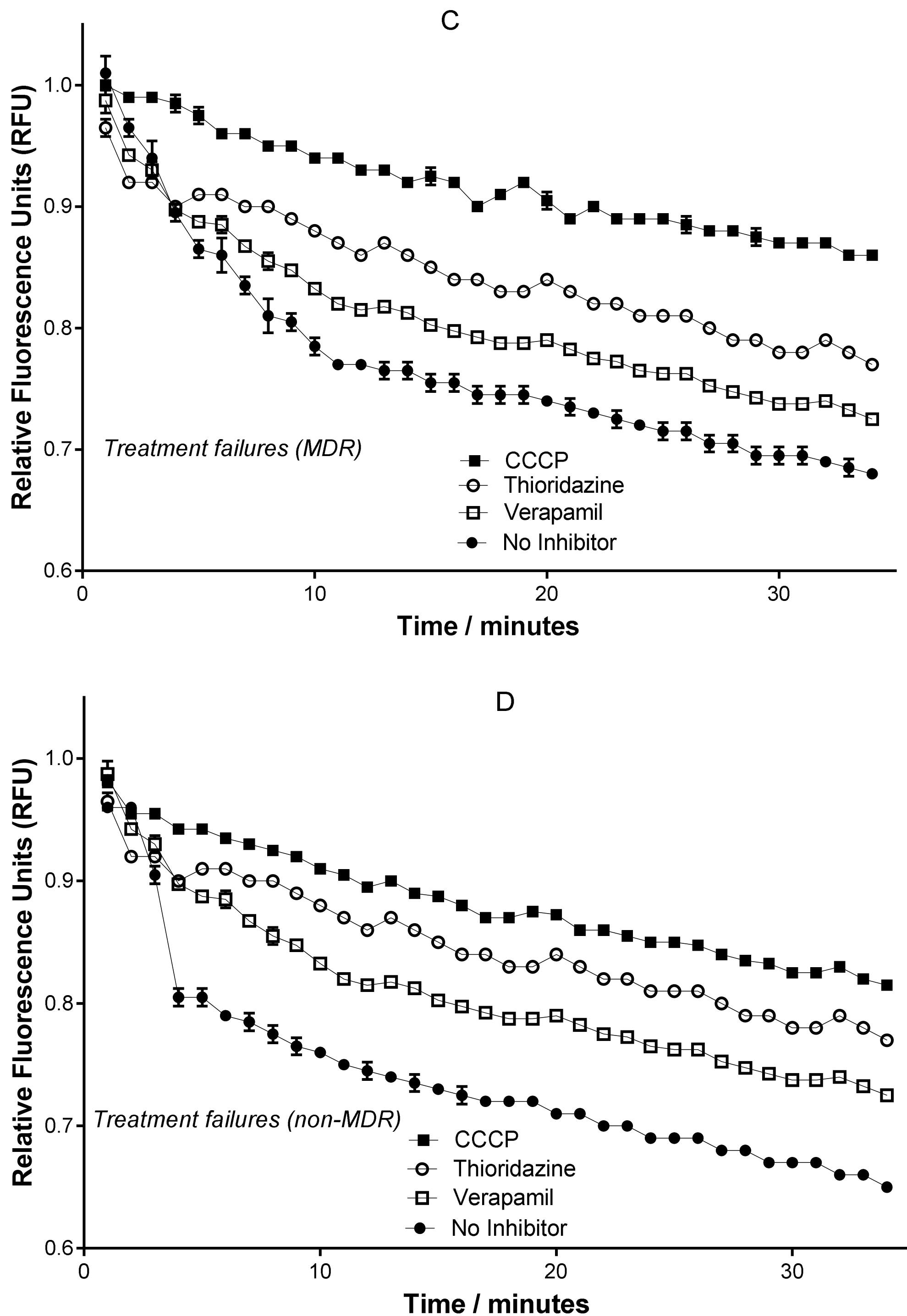

Figure 2: Effect of efflux pump inhibitors CCCP (carbonyl cyanide m-chlorophenyl-hydrazone) at 40 µM thioridazine at 8 µg/ml and verapamil at 100 µg/ml on the accumulation of ethidium bromide at 1 µg/ml by *M. tuberculosis* strains; (A) H37Rv strain, (B) isolates from patients who did not responded to treatment, (C)isolates that were not MDR from patients who responded to treatment, and (D) isolates that were MDR from patients who responded to treatment.

Quantification of expression of efflux pump genes by Real Time quantitative PCR (RT-qPCR): This Study analysis demonstrated that suboptimal levels of INH and RIF induced differential expression of the *Rv1819c* efflux pump that was expressed more significantly in treatment failures than in treatment successes and the H37Rv strain (Table 4). Most studies to date have used either drug but never in combination. The *Rv1819c* efflux pump was expressed three times as much in clinical isolates compared to the standard H37Rv strain. Expression of the *Rv1819c* pump in isolates from treatment failures was significantly ($p \leq 0.05$ using the Mann-Whitney U Test) higher than in isolates from treatment successes (Table 4). The findings of this study are consistent with findings that *Rv1819c* was over expressed only upon isoniazid exposure (Jiang *et al.*, 2008). Table 5 shows that the odds ratio for the association between treatment failure and *Rv1819c* expression was 0.49.

Table 5: Logistic regression analyses for the association between efflux pump gene expression and treatment failure.

Gene	Odds Ratio	P > |z|	Std. Err.	Regression Coefficient	Prob > chi^2
Rv1819c	0.49	0.02	0.07	0.55	0.02

The analysis was done using Stata/MP 13.0 software.
*A new variable called outcome was generated coding treatment successes as equal to 0 and treatment failures as equal to 1.
The number of observation included all the individual reads, adding up to 180.
* indicates a **p** value that was not statistically significant at the 95% confidence interval used in the analysis

Multidrug resistant clinical isolates have been reported to display *Rv1819c* over expression upon isoniazid exposure alone (Sarathy *et al*., 2012). The up regulation of *Rv1819c* was associated with a fourfold increase in MIC of the TB drug mefloquine (Danelishvili *et al*., 2005). Our previous study confirmed the role of *Rv1819c* protein in effluxing ciprofloxacin when over expressed in *C. glutamicum* (Mazando *et al*., 2013). The *Rv1819c* protein is homologous to the BacA family of proteins. Heterologous expression of the *M. tuberculosis* BacA homolog in *Escherichia coli* conferred sensitivity to antimicrobial peptides (Domenech *et al*., 2009). BacA-related proteins have been implicated in the transport of a critical molecule that determines the outcome of *M. tuberculosis* host-pathogen interaction (Mazando *et al*., 2013). However, attempts for the construction of deletion mutants *Rv1819c*, have been were unsuccessful for reasons not yet determined (Li *et al*., 2004, Chang *et al*., 2015). Our study has shed more light on the involvement of *Rv1819c* in clinical isolates thus highlighting the importance of this efflux pump concerning TB drug resistance.

CONCLUSION

In conclusion, in addition to classical mutations, the *Rv1819c* efflux may have a role in TB drug tolerance. This study found that high *Rv1819c* efflux pump activity and expression at baseline can be associated with tuberculosis treatment failure even when the *M. tuberculosis* does not have established resistance mutations. We, however acknowledge that we were unable to mitigate the effects of baseline bacterial load and host (patient) genetic factors as confounders of treatment outcomes.

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Appendix

Table 1: Gene mutations conferring RIF resistance on the 20 MDR isolates as identified by the MTBDRplus assay.

Number of isolates	**MTBDRplus assay**		
	RIF Phenotype	Mutation detected	Mutation or mutated codon (*rpoB*)
1	Resistant	ΔWT8	530–533
3	Resistant	WT, MUT3	S531L
1	Resistant	WT, MUT2A	H526Y
1	Resistant	ΔWT7	526–529
1	Resistant	ΔWT2	510–513
3	Resistant	ΔWT2, ΔWT3	510–517
4	Resistant	ΔWT8, MUT3	S531L
4	Resistant	WT, MUT2A, MUT3	H526Y and S531L
2	Resistant	ΔWT7, MUT2A	H526Y

ΔWT, wild-type band pattern missing, WT, sample had all wild-type bands present, MUT, sample had band indicating a mutation

Table 1: Gene mutations conferring INH resistance on the 20 MDR isolates as identified by the MTBDRplus assay.

Number of isolates			**MTBDRplus assay**		
	RIF Phenotype	**INH pattern (*katG*)**	**INH pattern (*inhA*)**	**Mutation or mutated codon (*katG*)**	**Mutation or mutated codon (*inhA*)**
3	Resistant	WT, MUT1	WT	S315T	WT
1	Resistant	ΔWT, MUT1	WT	S315T	WT
2	Resistant	WT	ΔWT1, MUT1	WT	C15T
1	Resistant	ΔWT	WT	315	WT
3	Resistant	ΔWT	ΔWT2	315	T8C
4	Resistant	ΔWT	ΔWT1, MUT2	315	C15T
3	Resistant	WT	MUT2	WT	T8C
3	Resistant	WT	ΔWT2	WT	T8C

ΔWT, wild-type band pattern missing, WT, sample had all wild-type bands present, MUT, sample had band indicating a mutation