Journal of Medical and Biomedical Sciences (2017) 6(1): *8-17*

doi: http://dx.doi.org/10.4314/jmbs.v6i1.2

ORIGINAL ARTICLE

High efflux pump activity and gene expression at baseline linked to poor tuberculosis treatment outcomes

S. Mazando[1], C. Zimudzi[1], M. Zimba[1], S. Sande[1], M. Gundidza[2], J.H. Mazorodze[3], P.M.M. Seepe[3], A. Pym[3] and P. Mason[4]

[1]Biological Sciences Department, University of Zimbabwe, 630 Churchill Avenue [2]Phamaceutical Technology Department, Harare Institute of Technology, Belvedere, [4]Biomedical Research and Training Institute, Avondale, Harare, Zimbabwe, [3]KwaZulu-Natal Research Institute for Tuberculosis and HIV (K-RITH), K-RITH Tower Building, level 3, Nelson R. Mandela School of Medicine, 4001 Durban, South Africa.

Phenotypic TB drug resistance, also known as drug tolerance, has been previously attributed to slowed bacterial growth *in vivo*. The increased activity and expression of efflux systems can lower the intracellular concentration of many antibiotics thus reducing their efficacy. We hypothesized that efflux pump activation and expression could be a risk factor for TB drug tolerance in patients initiated on treatment. Analyses of gene expression levels of six select efflux pumps associated with drug tolerance in *Mycobacterium tuberculosis* and its correlation with the cell's ability to efflux ethidium bromide (a common efflux substrate) were assayed. Efflux pump gene expression differed significantly between the strains from treatment failures and treatment successes. Efflux of ethidium bromide by *M. tuberculosis* isolates revealed that isolates from treatment failures rapidly efflux ethidium bromide more than isolates from treatment successes or the H37Rv control strains. The efflux pumps efpA, *jefA (Rv2459c)*, *Rv1258c*, *p55* and *mmpL7* may have a role in TB drug tolerance. Quantifying the expression levels of *M. tuberculosis* efflux pump genes may be a new method to diagnose clinically persistent tuberculosis. High efflux pump activity and expression at baseline can be associated with tuberculosis treatment failure even when the *Mycobacterium tuberculosis* does not have established resistance mutations.
Journal of Medical and Biomedical Sciences (2017) 6(1), 8-17

Keywords: drug resistance, Efflux, *Mycobacterium tuberculosis*, expression, treatment outcome

INTRODUCTION

Tuberculosis (TB) remains a serious public health threat around the world, and according to the World Health Organization, nearly two billion people were infected with *Mycobacterium tuberculosis*, with about 10.4 million of new TB cases, 1.4 million deaths among HIV-negative cases of TB and an additional 0.4 million deaths among people who were HIV-positive (WHO, 2016). Moreover, the emergence of multidrug resistant tuberculosis (MDR-TB) represents a challenge to the control of the disease since more than 480,000 of the TB cases in 2015 were estimated to be MDR-TB (WHO, 2016). Most of the estimated number of cases in 2010 occurred in Asia (59%) and Africa (26%). Existing TB control measures have proven inadequate to control the spread of TB in an HIV co-infected population (Amaral *et al.*, 2010).

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 two days of treatment, after which the rate of killing drops off markedly. 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 (Calver *et al.*, 2010). The

Correspondence: *Sungai Mazando, Biological Sciences Department, University of Zimbabwe, 630 Churchill Avenue, Mount Pleasant Harare, Zimbabwe*
Email: sungimazando@gmail.com

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 (Szumowski *et al.*, 2012). 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.

This study probes whether efflux pump activation and expression is associated with clinical persistence of TB bacilli in patients initiated on treatment. We hypothesized that efflux pumps could be a risk factor for tolerance to TB drugs such that *M. tuberculosis* is not rapidly cleared in patience during the intensive phase of TB treatment. The increased expression of efflux systems can significantly lower the intracellular concentration of many antibiotics thus reducing their clinical efficacy (Singh *et al.*, 2011). The efflux pumps chosen for this study include the pumps of ATP Binding Cassette (ABC) transporters (*p55*), Major Facilitator Superfamily (MFS) family (*efpA*, *Rv2459* and *Rv1258c*), Resistance Nodulation Division (RND) family (*mmpL7*) and Small Multidrug Resistance (SMR) family (*mmr*).

Analysis of gene expression of the six 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 if clinically persistent TB strains demonstrate increased efflux activity and expression compared to susceptible strains. We further demonstrate that drug tolerance can be reverted by efflux inhibitors, supporting their role as potential adjuvants in anti-tuberculosis therapy and prevention of clinical persistence.

MATERIALS AND METHODS

Study design

The study employed a retrospective case study recruiting isolates from patients who had not responded to normal Directly Observed Treatment Short-Course (DOTS) treatment as evidenced by a smear positive sputum sample after two months of treatment as cases.

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.

M. tuberculosis isolates

Frozen mother cultures of TB isolates (60) from a Mycobacterial Repository at the Biomedical Research and Training Institute National reference laboratories, which were deposited in the repository from July 2005 through January 2015 and which could be matched to a patient with a known treatment history were used in this study. The isolates were conveniently selected from patients who presented with clinical symptoms of TB and had not received TB treatment before. The sex ratio was also balanced out. Of the 60 isolates, 40 isolates were cases and 20 isolates were from patients who had responded to treatment within the first two months of treatment and served as controls. Of the 40 cases, 20 appeared genetically susceptible while 20 were multi drug resistant as confirmed by GeneXpert MTB/RIF test, GenoType MTBDRplus test and by Lowenstein-Jensen (L-J) culture method.

MIC determination and antibiotic susceptibility testing

Drug susceptibility testing (DST) was performed on Lowenstein–Jensen Medium using the proportion method. Minimum inhibitory concentrations (MICs) were defined as the lowest concentration of drug that inhibits growth of more than 99.0% of a bacterial population of the tested *M. tuberculosis* within 14 to 28 days of incubation at 37°C (Schönfeld *et al.*, 2012). Serial drug concentrations of the tested drugs were incorporated before inspissation. MIC was defined as the drug concentration on which <20 colonies (equivalent to 1% critical proportion) were observed, while confluent growth was observed on the drug-free medium. Reference strain H37Rv was used as an internal control for each batch of medium produced.

Assay of ethidium bromide accumulation and efflux in intact cells

The detection of ethidium bromide accumulation and efflux by the *M. tuberculosis* isolates on a real-time basis was performed using a fluorometric method previously described by Rodrigues *et al.* (2012) with minimum alterations. Mycobacterial cells were cultivated at 37°C for 2 to 3 days in 7H9 medium supplemented with Tween 80 and OADC to mid-exponential phase (OD_{600}, ca. 0.8 to 1.0), the cells were harvested at 5,000 g for 5 minutes at room temperature, washed once with 50 mM sodium phosphate buffer (pH 7.2), and re-suspended in the same buffer at an OD_{600} of 0.5. Aliquots of 100 µl of bacterial suspension were transferred into a 96-well plate containing serial dilutions of ethidium bromide at concentrations ranging from 10 to 0.325 µg/ml.

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 half the MIC in order to not 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. High RFU values indicated that cells accumulated more ethidium bromide under the tested conditions than under the reference conditions. The experiments were repeated three times, and the RFU values presented are the averages of three independent assays.

For assaying efflux, conditions that were shown to cause maximum accumulation of ethidium bromide without causing any significant inhibition of growth, as confirmed by cfu counting, were used for the loading of *M. tuberculosis* isolates. The following were the selected conditions: accumulation at 30 °C in the absence of glucose; use of an ethidium bromide concentration that caused a higher accumulation without compromising the cellular viability (3 mg/L). The 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 re-suspended in the same buffer 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.

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, relative to the ethidium bromide loaded cells.

Genotypic characterization of the strains

Mutations in the rpoB operon were screened on all isolates, using the system GeneXpert MTB/RIF test (Cepheid GeneXpert® System) and GenoType MTBDRplus test (Hain Lifescience) according to the manufacturer's instructions.

Quantification of expression of gene coding for efflux pumps by RT-qPCR

All 60 *M. tuberculosis* strains were sub-cultured in 7H9 medium with OADC supplement plus sub inhibitory concentrations of both INH and RIF at ¼ 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 re-

verse primers are listed in Table 2.

RT-qPCR assay

The relative expression level of genes that code for seven membrane efflux transporters in *M. tuberculosis (p55, Rv1819c, mmpL7, efpA, mmr, Rv1258c and Rv2459)* were 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 *i*Taq™ universal SYBR® Green one-step kit (Biorad). The determination of the relative mRNA expression level was performed using the comparative quantification cycle (Cq) method (Livak and Schmittgen, 2001). The relative expression of the efflux pump genes was assessed by comparison of the relative quantity of the respective mRNA in the H37Rv control strain. Each culture was assayed in triplicate using total RNA obtained from three independent cultures. A level of relative expression equal to 1 indicates that the expression level was identical to that of the H37Rv control strain.

Statistical Analysis

Data was entered into Microsoft excel and exported to SPSS version 20.0 for analysis. Figures and tables was used to describe data. Inferential statistics such as unpaired t-test and Wilcoxon Signed-Rank Test using both the Z-score and the W-value was used to inferred the data. P-value < 0.05 was considered statistically significant.

RESULTS

For 115 (64%) of the 180 MIC tests carried out in this study, results were available by day 21of incubation and by day 28 for the remaining tests. As shown in Table 1, the MICs for the clinical isolates were generally higher than for H37Rv control strain as was expected (Machado *et al*., 2012). The MICs of isolates from patients who had responded to treatment were significantly lower (P values <0.05 as indicated by ↕), than isolates from patients who had not responded to treatment using the unpaired t-test. Isolates that had been characterised as MDR by GeneXpert® System showed resistance not only to rifampicin but every other drug except for ethionamide.

Table 1: Minimum inhibitory concentration (MIC) determination and susceptibility testing for the strains exposed to different drugs in the presence and absence of efflux pump inhibitors (EPIs)

	INH			RIF			EMB			LFX			CFX			ETH		
ISOLATE DESCRIPTION	No EPI	+CCCP	+Ver	No EPI	+CCCP	+Ver	No EPI	+CCCP	+Ver	No EPI	+CCCP	+Ver	No EPI	+CCCP	+Ver	No EPI	+CCCP	+Ver
H37Rv	0.2	0.18	0.13*	2.25	0.75*	0.88*	1.5	0.90*	1.03*	0.88	0.58*	0.47*	1.25	0.50*	0.68*	5	3.50*	4.25*
Responded to treatment	0.25	0.24	0.24	9.92	8.71*	8.10*	2.69	1.93*	1.41*	2.85	1.81*	0.98*	1.41	0.75*	0.88*	11.2	9.11*	8.98*
Did not respond to treatment																		
Non-MDR isolates	0.32↕	0.23*	0.28* 0.88↕	12.6↕	4.73*	6.09*	5.11↕	1.67*	2.07*	3.00↕	0.91*	1.21*	1.60↕	0.59*	0.66*	19.3↕	6.86*	8.04*
MDR-Isolates	1.05↕	0.75↕*	*	≥40↕	20.3*	15.3*	7.50↕	5.44*	5.19*	4.07↕	0.95*	1.48*	2.38↕	0.94*	0.85*	31.0↕	29	30.1

MIC was defined as the drug concentration on which <20 colonies (equivalent to 1% critical proportion) were observed. The MIC values were calculated as an average of 20 isolates, each tested in triplicate. Efflux pump inhibitors (EPIs) were used at 1/2 of their MICs. MICs for the EPIs against H37Rv control strain: verapamil (Ver): 200 mg/ml, CCCP (carbonyl cyanide m-chlorophenyl-hydrazone); 40 µM.
↕ significant difference($p \leq 0.05$) between isolates from patients who did not respond to treatment when compared with isolates from patients who responded to treatmentusing the unpaired t-test
**** isolates whose MICs changed significantly ($p \leq 0.05$) in the presence of efflux pump inhibitors when compared without inhibitors using the unpaired t-test***

Notable decreases in the MICs of all drugs were observed in the presence of the efflux pump inhibitors verapamil (Ver): 200 mg/ml, carbonyl cyanide m-chlorophenyl-hydrazone (CCCP); 40 μM, (P values <0.05 as indicated by *), suggesting that the resistance levels could be affected by exposure to efflux pump inhibitors. The compounds selected have established inhibitory activity against mycobacterial efflux pumps (Rodrigues *et al.*, 2011). Sensitivity to INH was fully restored (MIC <0.25 μg/mL) to levels equal or below the critical concentration used for the standard susceptibility testing in the presence of efflux pump inhibitors in all non-MDR isolates including those from patients who had not responded to treatment. However, sensitivity to isoniazid and ethambutol was not restored even in the presence of efflux pump inhibitors in MDR isolates. Taken together, the data supports that efflux plays a critical role in the phenomenon of drug resistance.

The real time analysis of relative expression of six efflux pump genes that have been previously identified as transporters (Bianco *et al.*, 2011; Szumowski *et al.*, 2012; Li *et al.*, 2015b) of different anti-tubercular drugs was performed for all isolates as detailed in the methods section. Efflux pump gene expression differed significantly between the treatment failures and treatment successes according to the Wilcoxon Signed-Rank Test using both the Z-score and the W-value at $p \leq 0.05$. Only *mmr* gene did not show significant differences between the treatment failures and treatment successes. Efflux pump gene expression between the drug resistant and non-drug resistant isolates from treatment failures were not significant (Table 2).

Several strategies and experimental methods quantify the activity of efflux pump systems using measurement of fluorescent, radiolabelled or metal-labelled substrates. However, these methods, for the most part have failed to separate accumulation of substrate from its extrusion and vice versa (Rodrigues *et al.*, 2011). In this study an automated method was employed that demonstrates and quantifies efflux pump activity on a real-time basis, therefore allowing the identification of specific efflux pump inhibitors

Table 2: Relative expression levels ($2^{-\Delta CT}$value) of six drug efflux genes of clinically persistent and drug sensitive *M. tuberculosis* isolates

			Did not respond to treatment					
			Responded to treatment		Non-drug resistant strains		Drug resistant strains	
Gene	Efflux pump family	Primer Sequence	Median	25% – 75% value	Median	25% – 75% value	Median	25% – 75% value
mmpL7	RND	Forward:5' TGA AAT ACG GAA GCC TGG TC 3' Reverse:5' GAG GTA AGA GGC CAG CAC AC3'	0.23	0.15 – 0.44	0.33*	0.18 – 0.53	0.31*	0.17 – 0.48
Rv1258c (tap)	MFS	Forward:5' GGT ATG CCG TGT TGG CTA TC 3' Reverse:5' CCG CGT CTG TAT CAC GTA GTT 3'	0.95	0.53 – 0.97	1.43*	0.85 – 2.47	1.30*	0.60 – 3.14
Rv1410c (P55)	ABC	Forward:5' AGT GGG AAA TAA GCC AGT AA 3' Reverse:5' TGG TTG ATG TCG AGC TGT 3'	1.06	0.35 – 1.98	1.46*	0.40 – 2.27	1.22	0.53 – 2.58
efpA	MFS	Forward:5' TAG GTT TCA TCC CGT TCG TG 3' Reverse:5' TGA CCA GGT TGG GGA AGT AG 3'	2.9	2.48 - 3.41	5.16*	3.60 – 7.05	4.11*	3.20 – 6.34
Rv2459c (jefA)	MFS	Forward:5' TGA TCT TTC TCG ACG CAC TG 3' Reverse:5' CAG CGT GAA CAA CGA AAC AC 3'	2.65	1.94 – 4.90	4.04*	2.72 – 5.19	4.00*	2.16 – 5.79
mmr	SMR	Forward:5' AAC CAG CCT GCT CAA AAG 3' Reverse:5' CAA CCA CCT TCA TCA CAG A 3'	0.35	0.170 – 0.50	0.42	0.25 – 0.54	0.38	0.30 – 0.57

The relative expression of the efflux pump genes was assessed by comparison of the relative quantity of the respective mRNA in the H37Rv control strain. Each culture was assayed in triplicate using total RNA obtained from three independent cultures.
**** Efflux pump gene expression that differ significantly between the treatment failures and treatment successes according to the Wilcoxon Signed-Rank Test using both the Z-score and the W-value at $p \leq 0.05$.***

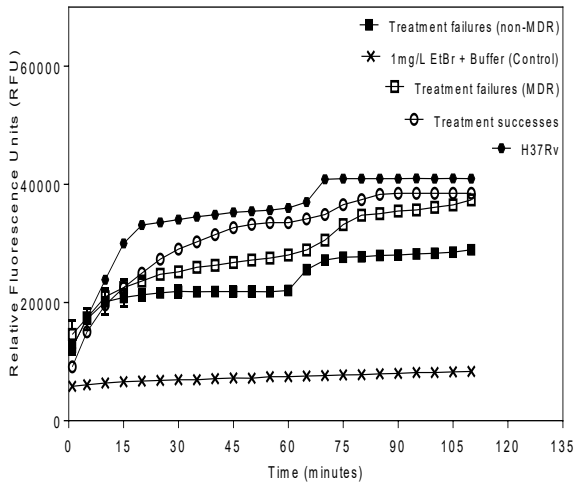

Figure 1: Accumulation curve of EtBr by the *M. tuberculosis* isolates tested over time when incubated at 37 °C in 1 µg/mL EtBr. After 60 minutes an efflux pump inhibitor CCCP (carbonyl cyanide m-chlorophenyl-hydrazone) was added at 40 µM. Data shown represent the means of the results from three separate experiments in which triplicate samples were taken at each time point

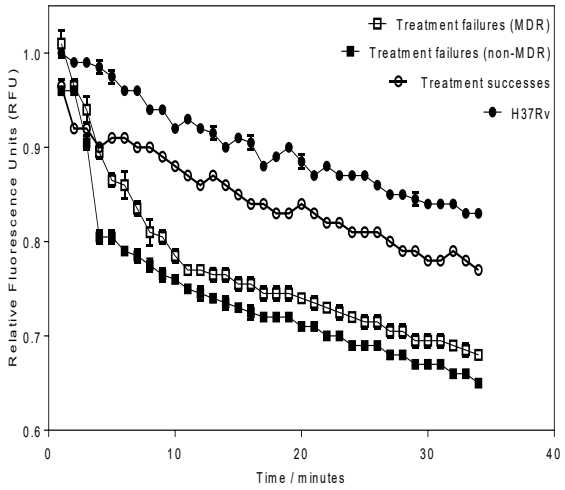

Figure 2: Efflux of ethidium bromide by *M. tuberculosis* isolates. The assay was conducted at 37 °C with glucose, after loading the mycobacteria with ethidium bromide at 5 mg/L. Data shown represent the means of the results from three separate experiments in which triplicate samples were taken at each time point.

Table 3: Relative fluorescence units (RFU) based on the accumulation of ethidium bromide for TB isolates in the absence and presence of efflux pump inhibitors (EPI)

Isolate description	No EPI	Log $_{10}$ RFU for the following efflux inhibitor		
		CCCP	Verapamil	Thioridazine
H37Rv	4.56 ± 0.03	4.67 ± 0.04®	4.58 ± 0.06	4.60 ± 0.03®
Responded to treatment	4.52 ± 0.04*	4.59 ± 0.04®	4.50 ± 0.04	4.60 ± 0.02®
Did not respond to treatment (non-MDR)	4.34 ± 0.03*	4.48 ± 0.02®	4.47 ± 0.02®	4.46 ± 0.03®
Did not respond to treatment (MDR)	4.49 ± 0.01*	4.57 ± 0.02®	4.55 ± 0.02®	4.58 ± 0.05®

The values correspond to the last point of measurement at 60 min, when fluorescence had reached a steady state. Data correspond to the averages of three independent assays for twenty isolate descriptions.
****isolates whose RFU is significantly different (p≤0.05) from the H37Rv strain using the unpaired t-test***
® RFU values that differ significantly (p≤0.05) in the presence of efflux pump inhibitor when compared without an efflux pump inhibitorusing the unpaired t-test

(EPIs).

As shown in Figure 1, accumulation of ethidium bromide reached steady state within thirty minutes when incubated at 37 °C in 1 µg/mL EtBr. The accumulation of ethidium bromide was significantly less in isolates from patients who had failed treatment compared to the control H37Rv strain (Table 3).

Thioridazine, CCCP and verapamil significantly increased the accumulation of ethidium bromide in all isolates including the H37Rv control strain. Accumulation of ethidium bromide may be affected

by factors other than diminished efflux pump activity such as increased permeability to ethidium bromide due to physical changes of the cell envelope (Rodrigues *et al.*, 2011). It is essential to first employ conditions which promote the accumulation of ethidium bromide in assessing efflux of ethidium bromide and conditions that affect it. Efflux of ethidium bromide by *M. tuberculosis* isolates (figure 2) reveals that isolates from treatment failures rapidly efflux ethidium bromide more than isolates from treatment successes or the H37Rv control strain.

DISCUSSION

There have been several attempts to understand why TB drug treatment regimens require at least 6 months of combination chemotherapy. Previous research has implicated a small subpopulation of bacteria (that are genetically identical to susceptible cells) as capable of surviving even intensive phases of antibiotic therapy (Szumowski *et al.*, 2012). The mechanisms for how these "phenotypically tolerant" (sometimes called "persistent") bacteria survive are currently subject of much research. Understanding the mechanisms underlying such persistence in *M. tuberculosis* is critical to reducing the length of TB therapeutics and thus decreasing the TB burden globally.

In this study we used 20 isolates that were otherwise genetically drug susceptible but exhibited clinical persistence during chemotherapy of the patients they were isolated from. The resistance profiles of the isolates were characterised by GeneXpert MTB/RIF test and GenoType MTBDRplus test. It has been shown that mycobacterial strains can present an MDR phenotype as a consequence of increased activity of efflux pumps that prevent the compounds from reaching their intended targets (De Rossi *et al.*, 2006). The isolates used in this study were taken at the start of the DOTS therapy treatment course (at treatment baseline).

To test the hypothesis that increased efflux could be a risk factor for clinical persistence, we performed ethidium bromide accumulation assays of the isolates in the presence and absence of efflux pump inhibitors. There was significant difference in the accumulation of ethidium bromide between isolates from treatment failures that were drug susceptible (*clinical persistent*) and the rest of the isolates. The *clinical persistent* accumulated significantly less ethidium bromide due to a higher activity of efflux pumps.

High expression levels, mutations in or induction of efflux pumps has been reported to confer not only low level resistance but also clinically significant drug resistance (Rodrigues and Lockwood, 2011; Sarathy *et al.*, 2013). The presence of efflux pump inhibitors cccp, thioridazine and to a lesser extent verapamil caused significant changes in the intracellular concentrations of the drugs underscoring that efflux activity was responsible for the lower ethidium concentration observed for *clinical persisters*. The activity of most efflux pumps has been shown to be inhibited by several compounds such as verapamil, cccp and thioridazine (Amaral *et al.*, 2010; Gupta *et al.*, 2013; Aparna *et al.*, 2014). Most of the studies to date have involved the transfer of hypothetical efflux genes into a heterologous host (*Mycobacterium smegmatis* or *Mycobacterium bovis*) in demonstrating that overexpression of efflux genes increases resistance levels (Gupta *et al.*, 2010; Li *et al.*, 2015a) whereas fewer have investigated active efflux in clinical isolates (Gupta *et al.*, 2010; Li *et al.*, 2015b).

Only a subpopulation of intracellular bacteria exhibits antibiotic tolerance at any given time in a bacterial population, likely implying that there is variation in efflux pump expression, with higher-expressing organisms attaining a drug-tolerant, macrophage growth-adapted phenotype (Szumowski *et al.*, 2012). To identify the efflux system(s) involved in the clinical persistence, we selected a set of genes coding for efflux pumps reported to be involved in the transport of various drugs (Machado *et al.*, 2012). Our analysis demonstrated that suboptimal levels of INH and RIF induced differential expression of some of these genes including *efpA*, *jefA*, *Rv1258c*, *p55* and *mmpL7* which were expressed more significantly in treatment failures than in treatment successes and the H37Rv strain. Previous studies have shown that *efpA* and *jefA* are overexpressed under isoniazid

stress (Waddell *et al.*, 2004; Machado *et al.*, 2012; Gupta *et al.*, 2013; Li *et al.*, 2015b). *P55* is functionally connected to processes that involve preservation of the cell wall and transport of toxic compounds away from the cells (Bianco *et al.*, 2011).

MmpL7 mediate transport of important cell wall lipids across the mycobacterial membrane and is also involved in other processes such as drug efflux (Edward *et al.*, 2013; Bailo *et al.*, 2015). *Rv1258c* expression in *M. tuberculosis* is under the transcriptional control of *WhiB7* transcriptional regulators. *WhiB7* is induced in response to sub-inhibitory concentrations of antimicrobials and mediates *Rv1258c* transcription in these settings (Szumowski *et al.*, 2012). The differences between isolates from treatment failures and treatment success suggest that quantifying the expression levels of *M. tuberculosis* efflux pump genes may be a new method to diagnose clinically persistent tuberculosis (Li *et al.*, 2015a). Mining through published literature on efflux pumps has revealed that 19 of the 55 annotated efflux pumps in the *M. tuberculosis* genome are transcriptionally-induced in macrophages (Szumowski *et al.*, 2012). The conservation of these efflux pumps in a wide range of pathogenic mycobacteria points to their inhibition as a therapeutic strategy not only for TB but for other difficult to treat mycobacterial diseases.

Our study reveals the presence of drug-resistant isolates that are clinically persistent which have no identifiable mutations in known resistance-associated genes, and it appears that resistance in these isolates results from increased efflux activity. Multiple studies have reported increased efflux pump expression in clinical isolates showing that a subpopulation of bacteria becomes tolerant to multiple classes of antimicrobials including isoniazid and rifampicin upon intracellular residence (Szumowski *et al.*, 2012). The activity of the efflux system(s) and their role in resistance to drugs was additionally confirmed by the use of efflux inhibitors in both real-time efflux assays and MIC determinations. MIC determination showed the involvement of efflux, as for drugs like isoniazid, the reduction of MIC by the efflux inhibitors reached levels identical to their susceptible counterparts particularly in isolates from treatment failures. This clearly shows that in these cases, high level contribution to resistance is mainly efflux-driven.

During the entire process, the clinical strains showed a capacity to handle higher drug concentrations than H37Rv, an additional evidence of their higher efflux capacity (De Rossi *et al.*, 2006; Balganesh *et al.*, 2010; Machado *et al.*, 2012). Overall, the clinical strains appear to be more prompt to respond to noxious agents via an efflux-mediated pathway, whereas H37Rv shows a less prompt use of efflux as a detoxifying response to drugs. The clinical implications of this phenomenon are quite serious. In most highly TB burdened nations where access to mycobacterial cultures are limited, the standard TB regiments prescribed to patients with unrecognized drug-resistant TB may not only have minimal efficacy but may serve to further limit treatment options. Thus, the addition of efflux pump inhibitors to overcome drug-tolerance may have the additional benefit of reducing emergence of both genetically detectable and phenotypic drug-resistant *M. tuberculosis*.

CONCLUSION

In conclusion, in addition to classical mutations, the efflux pumps *efpA*, *jefA*, *Rv1258c*, *p55* and *mmpL7* may have a role in TB drug tolerance. We have found that high 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. However, we acknowledge that we were unable to mitigate the effects of baseline bacterial load and host (patient) genetic factors as confounders of treatment outcomes.

ACKNOWLEDGMENTS

This work was supported by Grant number 2U2RTW007367 from the Fogarty International Centre, National Institutes of Health (NIH, USA) through the International Clinical, Operational and Health Services and Training Award (ICOHRTA).

COMPETING INTERESTS

The authors declare that they have no competing interests.

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WHO (2016) Global tuberculosis report 2016.

ISSN 2026-6294