EFFECT OF EFFLUX PUMP INHIBITORS ON RESISTANCE OF OFLOXACIN IN CLINICAL ISOLATES OF *MYCOBACTERIUM TUBERCULOSIS*

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ABSTRACT

Bacterial efflux pump systems contribute to antimicrobial resistance in pathogenic bacteria. The co-administration of bacterial efflux pump inhibitors with antibiotics overcomes the efflux mediated resistance to antibiotics. In this study, we assessed the effect of efflux pump inhibitors on resistance levels of ofloxacin by minimal inhibitory concentration (MIC) and analyzed the extent of the efflux pump mediated ofloxacin drug resistance in clinical isolates of *Mycobacterium tuberculosis*. A total of 50 *M. tuberculosis* clinical isolates were tested to observe the decrease in MIC by the resazurinmicrotitre assay method in the presence and absence of efflux pump inhibitors; verapamil, carbonyl cyanide m-chlorophenylhydrazone, chloropromazine, 2-4 di-nitro phenol & reserpine. In this study, inhibitors exhibit efflux activity both in resistant and susceptible isolates to ofloxacin. However, they are seen to be more active in resistant isolates, since the MIC of ofloxacin studied in these isolates was reduced between 2- and 8-fold. Reduction in MIC of ofloxacin was achieved by more than one efflux pump inhibitors. Our findings suggest that the involvement of active efflux pumps alone can exist as an independent mechanism of resistance in *M. tuberculosis* isolates apart of mutations in *gyrA*&*gyrB* genes.

Keywords: *Mycobacterium tuberculosis, fluoroquinolone, ofloxacin, efflux pump, efflux pump inhibitors*

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INTRODUCTION

The primary mechanism of fluoroquinolone (FQ) resistance in *Mycobacterium tuberculosis* is an alteration in DNA gyrase encoded by the *gyrA* and *gyrB* genes (Kocagoz *et al*., 1996, Takiff *et al*., 1994).Studies reported that the majority (approximately 50–90%) of FQ-resistant *M. tuberculosis* isolates carry mutations in the quinolone resistance-determining region (QRDR) of the *gyrA* gene (Yin *et al*., 2010, Brossier *et al*., 2010), and that a small number have mutations in the *gyrB* gene (Duong *et al*., 2009). As 10 - 50% of resistant strains lack mutations in the QRDR of the *gyrA* and *gyrB* genes, there must be other causes of FQ resistance in *M. tuberculosis* such as the efflux of drug molecules out of the mycobacteria (Siddiqi *et al*., 2002). Several known efflux pumps that confer resistance to one or several compounds have been described in mycobacteria (Paulsen *et al*., 1996). These efflux pumps include the pumps of Major facilitator superfamily (MFS) encoded by *lfrA*, *rv1634c&rv1268c* genes (Takiff *et al*., 1996, Liu *et al*., 1996)and ATP Binding Cassette (ABC) transporters encoded by *drrAB*, *pstB* genes and Rv2686c-2687c-2688c operon(Pasca *et al*., 2004). The natural function of efflux pump is the elimination of toxins from the cell. The upregulation of efflux systems by physiological induction and spontaneous mutation can significantly decrease the intracellular concentration of many antibiotics, reducing their clinical efficacy (Rossi *et al*., 2006). The effects of efflux pump inhibitors in reducing the resistance to antibiotics have been clearly shown in several bacteria (Aeschlimann *et al*., 1999). *In vitro,* the inhibition of active efflux in *M. tuberculosis* was observed in the presence of efflux pump inhibitors; carbonyl cyanide m-chlorophenylhydrazone (CCCP), verapamil (V), 2-4 di-nitro phenol (DNP) (Banerjee *et al*., 1996), reserpine, phenothiazines such as thioridazine (Kaatz*et al*., 2005, Rodrigues *et al*., 2011). The correlation of antibiotic resistance to an efflux pump has been observed when the level of MIC for antibiotic is lowerin the presence of efflux pump inhibitors (Choudhuri *et al*., 1999, Piddock *et al*., 2000, Silva *et al*., 2001). However, in the genome of *M. tuberculosis* a large number of putative genes that encode for efflux pumps are reported still, the studies related to the involvement of efflux pumps mediated FQ resistance in *M. tuberculosis* are limited. Therefore, the present study was planned to analyze the effect of efflux pump inhibitors on resistance levels of ofloxacin (OFX) and to analyze the extent of the efflux pump mediated OFX resistance in *M. tuberculosis* isolates.

MATERIALS AND METHODS

A total of 931 sputum samples from individual number of previously treated cases (received >4 weeks of anti-TB drugs in the past) of pulmonary TB were submitted for demonstration of Acid Fast Bacilli (AFB) and mycobacterial culture, during August 2012 to July 2013, at TB laboratory, Department of Microbiology, King George's Medical University, Lucknow, U.P., India.Total 670 isolates of *M. tuberculosis* were obtained from 931 samples and subjected to first-line [streptomycin (SM), isoniazid (INH), rifampicin (RIF) &ethambutol (EMB)] and second-line [ofloxacin (OFX)] drug susceptibility testing (DST) on solid Lowenstein Jensen media by 1% proportion method (Canetti *et al*., 1969). Critical concentrations were 0.2 µg/ml for INH, 4 µg/ml for SM, 40 µg/ml for RIF, 2 µg/ml for EMB, 2 µg/ml for OFX. A standard strain *M. tuberculosis* H37Rv was used as the quality control strain for each new batch of medium throughout the study.

Of the 670 *M. tuberculosis* isolates,100 OFX-resistant and 100 OFX-sensitive isolates of *M. tuberculosis* were consecutively selected for minimal inhibitory concentration (MIC) and

mutations in the QRDR of the *gyrA*&*gyrB* genes by sequencing,the data published in our previous study (Singh *et al*., 2014).Among 200 (100 OFX-resistant & 100 OFX-sensitive) *M. tuberculosis* isolates, 50 isolates were randomly selected to observe the decrease in OFX MIC in the presence and absence of efflux pump inhibitors, were grouped as: Group A; OFX-resistant with no mutations in the QRDR region of the *gyrA*&*gyrB* genes (21 isolates); Group B; OFX-resistant had mutations in the *gyrA* gene (14 isolates); Group C; OFX-sensitive with no mutation in the *gyrA*&*gyrB* genes (15 isolates). All the isolates were tested for OFX MIC by the Resazurinmicrotitre assay (REMA) method (Martin *et al*., 2009) with the concentrations of OFX (Sigma-Aldrich) ranging from 64-0.5 µg/ml, in the presence and absence of the following five efflux pump inhibitors i.e. verapamil (V), carbonyl cyanide m-chlorophenylhydrazone (CCCP), chloropromazine (CPZ), 2-4 di-nitro phenol (DNP) and reserpine (R). The solvent for V was distilled water and for CCCP, CPZ, DNP and R was dimethyl sulfoxide (DMSO). Each isolate was tested with each efflux pump inhibitor individually. All five inhibitors were procured from Sigma-Aldrich. The determination of OFX MIC in the presence & absence of efflux pump inhibitors was conducted by the broth micro dilution method, that they should not affect the growth of the control strain *M. tuberculosis* H37Rv and such a lowest concentration of each efflux pump inhibitors was chosen as final concentration for the study.

Minimal inhibitory concentration (MIC) by resazurinmicrotitre assay (REMA) plate method

The REMA plate method was carried out as described by Martin *et al*.,(2009) using sterile 96-well flat bottom plates. Plate setup for MIC of OFX in the presence and absence of efflux pump inhibitors is described in the figure. Briefly,100µl 7H9-S broth was dispensed in every well of sterile 96 well plates except blank (B) wells, where 200 µl of sterile distilled water was added to prevent evaporation during incubation. Two-fold serial dilutions of OFX were made directly into the wells, using volume of 100 µl working solution of OFX (256 µg/ml) prepared in 7H9-S medium.A total of 100 µl of diluted (1:10 ratio) bacterial suspension was added to each well except medium control and blanks. Final concentration of V (2.5 µg/ml), CCCP (1 µg/ml), CPZ (1 µg/ml), DNP (20 µg/ml) and R (20 µg/ml) was added in their respective wells. Medium control (MC) containing only medium; growth control (GC) containing medium and inoculum; and inhibitor control (IC) containing medium, inhibitor and inoculum were incubated in each plate. The plate was covered, sealed and incubated at 37°C. A stock solution of resazurin sodium salt powder was prepared at 0.02% in distilled water, filtered with 0.22 µm syringe membrane sterilized filter and kept at 4°C. After 7 days of incubation, 30 µl of resazurin solution was added to each well and the plate was re-incubated overnight. A change in color from blue (oxidized state) to pink (reduced state) indicated the growth of mycobacteria and the MIC was defined as the lowest concentration of each drug that prevented this change in color. The standard strain of *M. tuberculosis* H37Rv was tested as control (sensitive at <1 µg/ml levels of OFX). The OFX resistance and OFX susceptibility was defined as MIC of ≥4 µg/ml and MIC of ≤2 µg/ml, respectively. The assay for each strain was repeated thrice to validate the results. Two fold or more reduction in MIC levels was considered as presence of efflux activity in OFX-resistant *M. tuberculosis* isolates (Escribano *et al*., 2007, Singh *et al*., 2011). All the processing was necessarily done in biosafety level PIII.

RESULTS AND DISCUSSION

A total of 50 *M. tuberculosis* isolates showed decrease in OFX MIC,in presence of efflux pump inhibitors (Table 1).The OFX resistant isolates with and without mutation in the *gyrA* gene showed reduced OFX susceptibility although some of the isolates did not show any decrease in OFX MIC, in presence of efflux pump inhibitors. In OFX sensitive isolates (n=15), only 2-fold and 4-fold decrease in OFX MIC was observed, in presence of efflux pump inhibitors (Table1). AmongOFX resistant isolates with *gyrA* gene mutations (n=14), 11 (78.6%) isolates showed decrease in OFX-MIC and remaining 3 (21.4%) isolates,no decrease in OFX-MIC was observed. Of the 11OFX resistant isolates inhibited by all five inhibitors, 3 isolates were phenotypically changed their OFX MIC from resistant (≥ 4 µg/ml)to sensitive (≤ 2 µg/ml) in the presence of efflux pump inhibitors (Table 2). The MIC reduction in 42 (84%) isolates was observed by all five efflux pump inhibitors (V, CCCP, CPZ, DNP & R) however, 8 (16%) isolates (3 isolates of OFX-sensitive and 5 isolates of OFX-resistant) did not show anydecrease in OFX MIC. Reduction in MIC of OFX was achieved by more than one efflux pump inhibitor (Table 3).The role of efflux pumps in conferring drug resistance has long been recognized in several bacteria. In mycobacteria, several other mycobacterial drug efflux pumps have been reported since the discovery of LfrA in *M. smegmatis*(Takiff *et al*., 1996, Liu *et al*., 1996). A drug accumulation study carried out in the presence and absence of an efflux pump inhibitors revealed small but reproducible activity of efflux systems towards RIF in *M. tuberculosis*, *M.aurum*, and *M. smegmatis*(Piddock *et al*., 2000).Singh *et al*., (2011) reported that 66.6% *M. tuberculosis* isolates showed inhibitory effect of efflux pump inhibitors on resistance levels of OFX MIC.

A study reported, the strains with reduced ciprofloxacin (MIC=2 mg/l) and linezolid (MIC=4 mg/l) susceptibility with no mutation in the *gyrA* gene, demonstrated the greatest activity of efflux systems (Escribano *et al*., 2007). Similarly, we found OFX resistant isolates with no mutation in the *gyrA* gene showed reduced OFX susceptibility in presence of efflux pump inhibitors. Although some of the isolates which were OFX resistant with no mutation in *gyrA* gene had also not shown any decrease in OFX MIC in presence of any of the five efflux pump inhibitors. This suggests that an alternative mechanism such as decreased cell permeability, drug sequestration or perhaps even drug inactivation(Drilca *et al*., 2003) play a role in conferring FQ resistance.

It has also been shown that FQ resistant *M. tuberculosis* strains with mutations in the *gyrA* and *gyrB* genes were influenced by the efflux pump inhibitor and showed two- and six fold decrease in the level of its resistance for OFX (Escribano *et al*., 2007). The OFX-resistant isolates with mutation in the *gyrA* gene had shown decrease in OFX MIC in presence of efflux pump inhibitors, revealing that both mechanisms (i.e. point mutation and efflux pumps) were involved in OFX resistance (Gupta *et al*., 2006). In presence of efflux pump inhibitors, the reversal of resistance to all major anti-tubercular drugs (SM, INH, RIF & OFX) is also reported, which vary in different mycobacterial species (Gupta *et al*., 2006)low activity of efflux pump inhibitors was reported in OFX-sensitive isolates (Singh *et al*., 2011).The OFX-sensitive isolates from the present study have shown only 2-fold and 4-fold decrease in OFX MIC, in presence of efflux pump inhibitors. It confirms the low activity of efflux pumps in OFX-sensitive isolates and the importance of active efflux systems, as described in other pathogens (Cebrian *et al*., 2003, Zhou *et al*., 2000).The present study revealed the role of efflux pump inhibitors in conferring OFX resistance in OFX-resistant isolates with no mutations in the QRDR region of the *gyrA* gene. Therefore, our findings suggest the

involvement of active efflux systems alone can exist as an independent mechanism of OFX resistance in *M. tuberculosis* isolates. It is essential to gain an insight into the mechanisms other than the mutations in their associated genes and over-expression of efflux pumps.

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Table 1: *Change in OFX MIC of M. tuberculosis isolates (n=50) in presence of efflux pump inhibitors*

S.no	Mutations in *gyrA* gene	Initial OFX MIC (µg/ml)	OFX MIC (µg/ml) in presence of inhibitors				
			V	CCCP	CPZ	DNP	R
1	Ala90Val	8	2	4	4	4	4
2		8	4	2	4	4	4
3		16	16	16	16	16	16
4		8	4	4	4	4	4
5		4	2	2	0.5	2	0.5
6		16	8	4	4	4	4
7	Asp94Gly	32	4	4	4	8	8
8		4	4	4	4	4	4
9		16	4	4	2	2	4
10		16	4	4	4	8	8
11		4	2	0.5	0.5	2	0.5
12		8	2	4	4	4	4
13	Asp94Asn	16	2	4	2	2	2
14	Ala90Val, Ser91Pro	16	16	16	16	16	16

[OFX, Ofloxacin; MIC, Minimal inhibitory concentration; V, Verapamil; CCCP, Carbonyl cyanide m-chlorophenylhydrazone; CPZ, Chloropromazine; DNP, 2,4 di-nitro phenol; R, Reserpine. A^a: OFX-resistant isolates without mutation in *gyrA* gene, B^b: OFX-resistant isolates with mutation in *gyrA* gene, C^c: OFX-sensitive isolates with no mutation in *gyrA* gene]

Table 2: *OFX-resistant isolates (n=14) with mutation in the gyrA gene of M. tuberculosis and change in their resistance levels of OXF MIC in presence of efflux pump inhibitors*

Groups	Efflux pump inhibitors	No. of isolates showed fold change in OFX MIC (%)			
		2 fold change	4 fold change	8 fold change	No change
A^a (n=21)	V	7 (33.3)	9 (42.8)	2 (9.5)	3 (14.2)
	CCCP	10 (47.6)	9 (42.8)	-	2 (9.5)
	CPZ	7 (33.3)	10 (47.6)	1 (4.7)	3 (14.2)
	DNP	11 (52.3)	4 (19)	2 (9.5)	4 (19)
	R	8 (38.1)	8 (38.1)	2 (9.5)	3 (14.2)
B^b (n=14)	V	5 (35.7)	4 (28.5)	2 (14.2)	3 (21.4)
	CCCP	4 (28.5)	5 (35.7)	2 (14.2)	3 (21.4)
	CPZ	4 (28.5)	2 (14.2)	5 (35.7)	3 (21.4)
	DNP	7 (50)	2 (14.2)	2 (14.2)	3 (21.4)

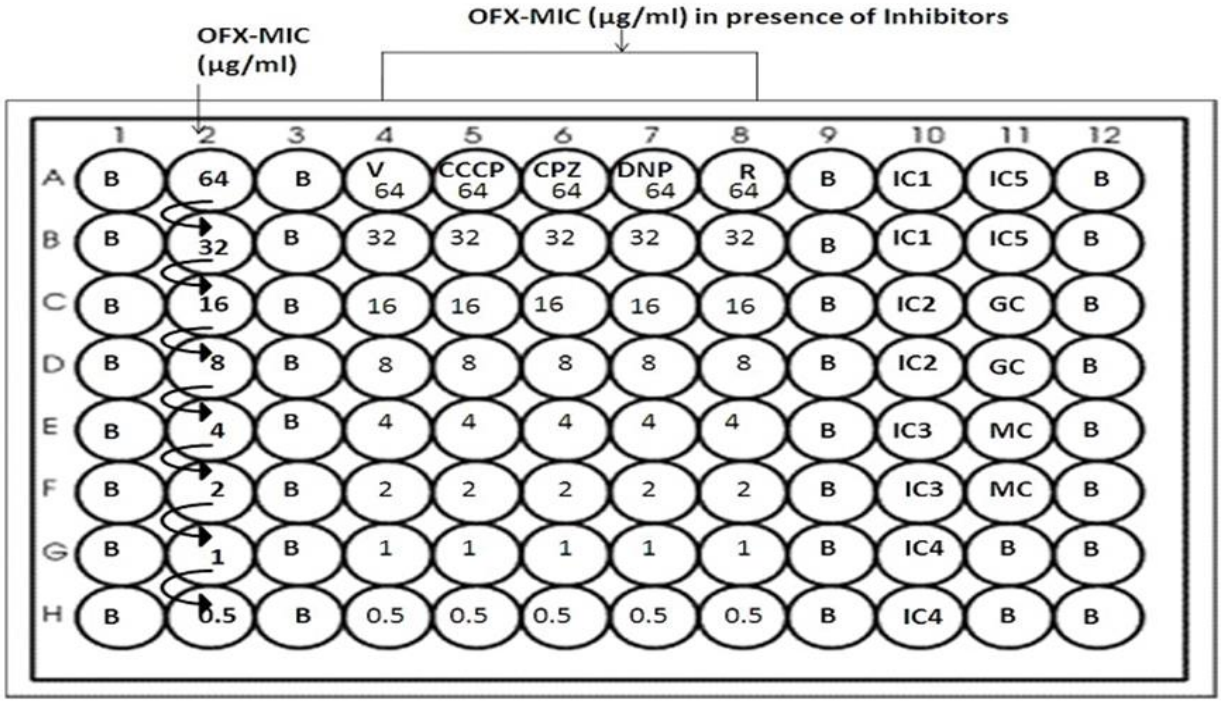
	R	5 (35.7)	3 (21.4)	3 (21.4)	3 (21.4)
C^c (n=15)	V	8 (53.3)	4 (26.6)	-	3 (20)
	CCCP	6 (40)	3 (20)	-	6 (40)
	CPZ	7 (46.6)	3 (20)	-	5 (33.3)
	DNP	7 (46.6)	3 (20)	-	5 (33.3)
	R	8 (53.3)	4 (26.6)	-	3 (20)

[OFX, Ofloxacin; MIC, Minimal inhibitory concentration; V, Verapamil; CCCP, Carbonyl cyanide m-chlorophenylhydrazone; CPZ, Chloropromazine; DNP, 2,4 di-nitro phenol; R, Reserpine]

Table 3: *Effect of more than one efflux pump inhibitors on resistance levels of OFX MIC in M. tuberculosis isolates*

	OFX MIC without efflux pump inhibitors (µg/ml)	**No. of isolates (n=50)**	**Decrease in OFX MIC observed in presence of more than one efflux pump inhibitors (no. of isolates)**
OFX-sensitive isolates	1	9	V, CCCP, CPZ, DNP & R (n=6)
			No reduction in MIC (n=3)
	2	6	V & R (n=2)
			V, CPZ, DNP & R (n=1)
			V, CCCP, CPZ, DNP & R (n=3)
OFX-resistant isolates	4	6	CCCP & R (n=1)
			V, CCCP, CPZ, DNP & R (n=3)
			No reduction in MIC (n=2)
	8	11	V, CCCP & CPZ (n=1)
			V, CCCP, CPZ, DNP & R (n=10)
	16	16	V, CCCP, CPZ, DNP & R (n=13)
			No reduction in MIC (n=3)
	32	2	V, CCCP, CPZ, DNP & R (n=2)

[OFX, Ofloxacin; MIC, Minimal inhibitory concentration; V, Verapamil; CCCP, Carbonyl cyanide m-chlorophenylhydrazone; CPZ, Chloropromazine; DNP, 2,4 di-nitro phenol; R, Reserpine]



[B, Blank; OFX, Ofloxacin; MIC, Minimal inhibitory concentration; MC, Medium control; GC, Growth control; IC, Inhibitory Control; V, Verapamil; CCCP,Carbonyl cyanide m-chlorophenyl hydrazine; CPZ, Chloropromazine; DNP, 2,4 di-nitro phenol; R, Reserpine]

* Drug concentration of OFX in each well is mentioned in the fig.

Column 1 A-H= Blank
Column 2 A-H= 7H9 broth + OFX + Inoculum
Column 3 A-H= Blank
Column 4 A-H= 7H9 broth + OFX + Inoculum + V (2.5µg/ml)
Column 5 A-H= 7H9 broth+ OFX + Inoculum + CCCP (1µg/ml)
Column 6 A-H= 7H9 broth+ OFX + Inoculum + CPZ (1µg/ml)
Column 7 A-H= 7H9 broth + OFX + Inoculum + DNP (20µg/ml)
Column 8 A-H= 7H9 broth + OFX + Inoculum + R (20µg/ml)
Column 9 A-H= Blank
Column 10 A-B (IC1)= 7H9 broth + Inoculum + V (2.5µg/ml)
C-D (IC2)= 7H9 broth+ Inoculum + CCCP (1µg/ml)
E-F (IC3)= 7H9 broth + Inoculum + CPZ (1µg/ml)
G-H (IC4)= 7H9 broth + Inoculum + DNP (20µg/ml)
Column 11 A-B (IC5)= 7H9 broth + Inoculum + R (20µg/ml)
C-D (GC)= 7H9 broth+ Inoculum
E-F (MC)= 7H9 broth
G-H= Blank
Column 12 A-H=Blank

Fig 1: *Plate setup for Minimal Inhibitory Concentration (MIC) in the presence and absence of efflux pump inhibitors by ResazurinMicrotite Assay (REMA)*

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