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Selection of m. tuberculosis clinically isolated sensitive & resistant to fluoroquinolones

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Article History	Abstract
<p>Received on: 05-01-2020 Revised On : 17-02-2021 Accepted on : 20-02-2021</p>	<p>In the present study we observed on performing docking analysis that OFX interacted with conserved residues of Usp, SDR, PspA and CoA transference domain of Rv2140c, Rv0148, Rv2744c and Rv3551, respectively, which might alter this function. It is predicted that these proteins might be exhibiting increased intensities to inhibit/modulate/compensate the effect of drugs. Further, detailed study in this direction might help to search new targets for drug development. Besides known proteins, upregulation of hypothetical proteins strengthen the possibility of some unknown underlying mechanism responsible for resistance to OFX. This could be crucial for the initial survival of the cells before gene level changes could come into play to ensure survival under prolonged adverse conditions. These findings may be further exploited to develop newer therapeutic agents derived from OFX. Further detailed and in-depth investigations to explore these leads will give an insight into probable sites of drug action, other than established primary sites and hence may help in the search of novel chemotherapeutic agents at these new sites as inhibitors and could provide the mankind with some ultimate treatment strategies.</p> <p><i>Keywords:</i> SDR, PsPA, OFX, MIC, EF-Tu, EF-P, E. Coli.</p>
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Introduction

Tuberculosis may be broadly defined as a chronic communicable bacterial disease caused by the members of genus Mycobacterium, usually M.tuberculosis but any member of the so called tuberculosis complex will also do. The WHO estimated 8.7 million (range, 8.5–9.2 million) incident cases of TB, 1.1 million (range, 0.9–1.2 million) deaths from TB among HIV-negative people and an additional 0.35 million (range, 0.32–0.39 million) deaths from HIV-associated TB. India accounts for one fifth of the global incidence with 1.9 million new cases and 0.3 million deaths. TB and HIV are frequently referred to as co- or dual- epidemics due to their high rate of co-infection, and TB is a leading cause of death among people with HIV, especially in developing countries. Increasing prevalence of TB makes this disease a major topic for vaccine research, novel drug targets and development of novel diagnostic tools [1].

Now-a-days drug resistance, particularly multi-drug resistance (MDR), extensively drug resistance (XDR), total drug resistance (TDR) and the TB-associated HIV and TB- associated with diabetes have emerged as major problems in the chemotherapy of tuberculosis. Despite significant progress, the number of MDR-TB cases notified in 2011 represented only 19% of the estimated 3,10,000 cases of MDR-TB among reported patients with pulmonary TB, and less than 10% in the two countries with the largest number of cases, China and India. Globally, 3.7% (2.1–5.2%) of new cases and 20% (13–26%) of previously treated cases are estimated to have MDR-TB. The average proportion of MDR-TB cases with XDR-TB is 9.0% (6.7–11.2%). In addition to HIV-TB co-infection, diabetes mellitus (DM) is now recognized as a major impediment to the successful treatment of TB infection. Persons with HIV and DM have a significantly increased risk (2-3X higher) of active TB. In countries like China and India, the prevalence of DM has increased

dramatically in recent years and the implementation of dual DM-TB screening efforts among local clinics has helped to identify cases of dualburden [2].

As of today, only vaccine available against *M. tuberculosis* is the attenuated strain of *M. bovis* known as Bacille Calmette Guérin (BCG), which is used in many parts of the world. Unfortunately, data suggest that while BCG vaccination can reduce the infancy tuberculosis, it appears ineffective at preventing the most common adult form of tuberculosis, pulmonary tuberculosis. Therefore, there is wide spread consensus that a new vaccine against tuberculosis is needed. This failure provides a thrust area for the development of newer vaccines (such as DNA and subunit vaccines), various trials of which are being conducted. GSK M72, a recombinant protein vaccine combined with a proprietary adjuvant AS01, is in phase IIb clinical trials [3].

Treatment of MDR-TB requires the administration of second line drugs (Aminoglycosides: kanamycin, amikacin, capreomycin; Fluoroquinolones: ofloxacin, ciprofloxacin, gatifloxacin; Macrolides etc) in higher doses, which are more toxic than the first line drugs. Extensively drug resistant TB (XDR-TB) is caused by infection of strains which in addition to MDR, are also resistant to one of the second line injectible drug (amikacin/kanamycin) and any one of the fluoroquinolones (ofloxacin/ciprofloxacin) and the type of infection is totally untreatable/incurable. The XDR-TB was first declared by WHO in 2006. In this situation, not only new chemotherapeutic agents, diagnostics, targets and new treatment regimen are required to treat patient; but also there is a need to enhance patient compliance by shortening the duration of treatment [4].

The increase of drug-resistant TB has stimulated interest in the understanding of the molecular mechanisms of drug resistance in *M. tuberculosis*, and significant progress has been made in this field. Non-availability of drugs, frequent lack of medical assistance and non-compliance of drug regimen are some of the reasons that have increased the incidence of drug resistance. Short course chemotherapy with Rifampicin (RIF), Isoniazid (INH), Pyrazinamide (PZA), Ethambutol (EMB) and Streptomycin (SM), being the backbone of the treatment, is the most powerful weapon available against mycobacterial infections which is now administered in the form of Directly Observed Treatment Short course (DOTS). However, dramatic outbreaks, caused by MDR strains of mycobacteria have focused international attention on drug resistance. Increase of drug-resistant TB has stimulated interest in the understanding of the molecular mechanisms of drug resistance in *M. tuberculosis* and to develop better drugs against *M. tuberculosis* [5].

The fluoroquinolones, introduced for clinical use in 1982, are the latest to join the chemotherapy of tuberculosis. However, we cannot lose sight of the fact that fluoroquinolones are highly useful for their broad spectrum effect for respiratory, genito-urinary and gastro-intestinal infections. The in vitro activities of fluoroquinolones against mycobacteria and the efficacy of these drugs in murine model of mycobacterial infection have been documented. The major fluoroquinolones are ciprofloxacin, ofloxacin, levofloxacin, sparfloxacin, gatifloxacin and moxifloxacin. As a consequence of the extensive and in some cases, indiscriminate use of fluoroquinolones, clinical resistance to the drug has been widely reported. Rapidly emerging resistance is an apparent drawback to their widespread use. The improper management of MDR-TB can result in further expansion in drug resistant profile and can lead to extensively drug resistant TB (XDR- TB) [6].

Although the primary mechanism of their action is by inhibiting gyrase, but other possible mechanisms cannot be ruled out. The mechanism of quinolone resistance involves primarily mutations in the A and B subunits of the DNA gyrase. The first site identified with fluoroquinolones (FQs) resistance mutation was a short segment of *gyrA* termed the Quinolone Resistance Determining Region (QRDR). Few FQ resistance mutations have also been described in *gyrB*, usually in a region of the protein thought to interact with the QRDR to form the FQ binding pocket. It is apparent that *M. tuberculosis* resistance to FQ occurs primarily due to mutation in the QRDR of *gyrA* gene. But only 42-85% of resistant *M. tuberculosis* isolates are have mutation in *gyrA* QRDR till date 14. No isolates have been found associated with *gyrB* QRDR mutation. Another mechanism of resistance to quinolones is the permeability barrier and efflux of the drug out of the cell. It is possible that fluoroquinolones may have some other mechanisms of action also. The studies carried out at NJIL&OMD have shown inhibiting effect of streptomycin, rifampicin and quinolones on mycolic acid biosynthesis in mycobacteria and ofloxacin has been found to inhibit the phenolic glycolipid biosynthesis in *M. bovis* BCG based on radiolabeled palmitate uptake. It is possible that these effects of quinolones on lipid biosynthesis is secondary to quinolones primary mechanism of action on gyrase or a primary event of low tune not given adequate attention so far. For better understanding of drug resistance, there is great need to find the exact mechanism of resistance in fluoroquinolones for *M. tuberculosis* (mutation, efflux pump or permeability barrier and others), which would help to find out the newer drug target/drug for better treatment of tuberculosis and various mechanisms contributing to FQ resistance in tuberculosis [7]. Several methods are used for analysis of gene expression. The oligonucleotide and cDNA microarrays and large-scale sequencing of expressed sequence tags quantitatively

measure gene expression at the mRNA level. Although that genomic analysis is informative, it only provides a limited view of dynamics associated with cellular responses to a particular stimulus or at steady state. Compared to mRNA studies, proteomic analysis provides more accurate assessments due to measurement of functionally relevant species. There is no direct correlation between mRNA expression and changes in stimulus in relation to post-translational control mechanism [13]. Proteomics, the global analysis of the proteins expressed in a cell or tissue, provides a very promising approach for the large scale identification of proteins, their complexes, and their functions. Pharmacoproteomics, i.e; "analysis of the pharmacological effects of drugs on the protein level", is a discipline that studies inter-individual variations in the proteins in conjunction with their pharmacological functions and therapeutic response. These studies can facilitate identification of new drug targets and provide information on the mode of action of new or existing drugs, which have traditionally remained unrecognized [8]. A combination of two-dimensional gel electrophoresis (2DE) and mass spectrometry (MS) is used for proteomics analysis and now the bioinformatics analysis has now emerged as a robust and efficient strategy for rapid identification and characterization of proteins. These studies are further aided by the availability of total genome sequence database. Proteome analysis of cytosolic and plasma membrane proteins of mycobacteria is well documented. Identification and mapping of culture filtrate proteins is also reported [12]. Proteins of isoniazid-resistant and susceptible strains of *M.tuberculosis* have been compared and this technique has also been used for studying susceptible and resistant strains of *M.tuberculosis*. MALDI- TOF MS has also been reported to be a rapid and reproducible method for the identification and characterization of Mycobacterium species. Recently analyzed the proteomes of secreted and cytosolic proteins of genetically closely related strains of *M. tuberculosis*. Comparative proteomic studies addressing whole cell proteins with first- and second-line aminoglycosides drug resistance isolates have been reported. Comparison of protein profile of Streptomycin induced SM mono-resistant isolate with SM sensitive isolate has already been reported, which revealed eight proteins found to be upregulated in the presence of drug 9,30. An isolated study by the effect of fluoroquinolone exposure on the proteome of *Salmonella enterica* serovar typhimurium showed 50% reduction in the number of proteins detected by 2D-Gel electrophoresis, derived from control cultures of wild-type *S. enterica* following treatment with ciprofloxacin 15. Upregulation of 17 proteins was detected in the above study. Up to 8-fold increase in the expression of subunits of F1F0-ATP synthase, TolC and Imp were observed. studied the pharmacoproteomic alterations of *M. tuberculosis* H37Rv strain, induced by antitubercular drugs isoniazid,

ethambutol and SQ109 (an ethambutol analogue), providing new understanding of pharmacoproteomic mechanisms of each drug and exploring new drug targets. We analyzed and compared the protein profiles of OFX, CFX and MOX induced total susceptible isolates and OFX induced OFX mono-resistant isolates with sensitive *M. tuberculosis* isolates. Such information could be helpful for the development of newer diagnostics and therapeutic agents for better treatment, particularly drug resistance TB10, [11].

Materials and methods

Mycobacterial isolates

Eleven *M.tuberculosis* isolates having different types of drug resistance profiles were included in the present study were obtained from the Mycobacterial Repository Centre of National JALMA Institute for Leprosy and Other Mycobacterial Diseases, Agra. All isolates were maintained on LJ medium and freshly sub-cultured before being used for further microbiological investigations [16,29].

List of Isolated mycobacterium

S.No.	Isolates	Identification	Type /Source	Susceptibility profile as on LJ medium (Resistant to)*
1.	H37Rv (TMC-102)	<i>M.tuberculosis</i>	Standard	Total sensitive
2.	Jal 2862	<i>M.tuberculosis</i>	Sputum	Total sensitive
3.	Jal 3211	<i>M.tuberculosis</i>	Sputum	Total sensitive
4.	Jal 3219	<i>M.tuberculosis</i>	Sputum	Total sensitive
5.	Jal 3265	<i>M.tuberculosis</i>	Sputum	Total sensitive
6.	Jal 3566	<i>M.tuberculosis</i>	Sputum	Total sensitive
7.	DKU317	<i>M.tuberculosis</i>	Sputum	Total sensitive
8.	Jal 3515	<i>M.tuberculosis</i>	Sputum	OFX
9.	Jal 3612	<i>M.tuberculosis</i>	Sputum	OFX
10.	Jal 3634	<i>M.tuberculosis</i>	Sputum	OFX
11.	OMR-CH	<i>M.tuberculosis</i>	Sputum	OFX

Drug susceptibility testing (DST) by proportion method

DST was performed for five first-line anti-TB drugs by conventional LJ proportion method. 4 ml standard bacterial suspension of 1 mg/ml in D/w was prepared by taking growth from LJ culture in a Bijou bottle containing glass beads and was homogenized by vigorous vortexing. Further, the suspension was serially diluted to 10⁻¹, 10⁻² and 10⁻³ mg/ml and used for inoculation on drug-free and drug-containing LJ slants with the help of a loop of 3 mm external diameter. Reference *M.tuberculosis* strain H37Rv was included as sensitive control for DST. All inoculated slopes were incubated at 37°C for six weeks to take the final readings. An isolate was

considered resistant if it yielded a growth of 1% or more on drug-containing LJ medium in comparison to the drug-free medium [28].

Determination of MICs of OFX, CFX & MOX

REMA plate method was performed to determine the MICs of all isolates against OFX, CFX and MOX. Briefly, 100 µl of 7H9 broth was dispensed in each well of a 96-well cell culture plate. OFX, CFX and MOX concentrations prepared directly in the medium were: 0.1, 0.2, 0.5, 1, 2, 4, 8, and 16 µg/ml. Perimeter wells were filled with sterile water to avoid dehydration of medium during incubation. Standard bacterial suspension of no. 1 McFarland standard was prepared, diluted 1:20 in 7H9 broth and 100 µl inoculum was used to inoculate each well. Growth control without drug and a sterile control without inoculum were also included for each isolate. Plates were covered with lid and incubated at 37°C for one week. 30 µl of 0.02% resazurin solution (blue) was added to each well and plates were reincubated for an additional 2 days. A change in color from blue to pink indicated the growth of bacteria. MIC was read as the minimum concentration of drug that prevented the colour change of resazurin solution [17,27].

Culture in Sauton's liquid medium and drug Treatment

All selected isolates were inoculated in culture tubes containing Sauton's liquid medium for adaptation of cultures from semi-solid medium to liquid medium and incubated for four weeks at 37°C. All the microbiological handling was performed in bio-safety cabinet following the standard microbiological practices for proper containment of biohazard materials. For large amount of growth, isolates were sub-cultured from culture tubes to large flasks (250 ml) containing Sauton's medium and incubated for four weeks at 37°C. After four weeks sub-inhibitory concentration of drugs were added to the cultures. The cells were harvested after 36 hrs considering generation time of 24-36 hrs for *M. tuberculosis* and growth was collected by centrifugation, washed with normal saline and stored at -20 °C for further use [18,26].

Preparation of mycobacterial whole cell lysate

Mycobacterial whole cell lysate was prepared according to. Growth pellets were suspended in sonication buffer (1 gm wet cell mass/ 5 ml) containing PMSF (1 mM), protease inhibitor cocktail (1 mM) and then broken by intermittent sonication for 20 min at 4°C in ice using sonicator. Homogenate was cleared by centrifugation at 12,000 g for 15 min x 2 times at 4°C. Pellets were discarded and supernatant was stored at -200C until used [19].

Protein precipitation with SDS-trichloroacetic acid (TCA)-acetone

Equal volumes of cell lysate were taken in three vials. Aliquot (a) was used for 2DE without protein precipitation while aliquots (b) and (c) were treated as follows: TCA (100% w/v) was added to aliquot (b) at final concentration of 10% (w/v) to aliquot (c); 10% SDS (w/v) was added to achieve final concentration of 0.1% (w/v) and after boiling for 2 min, TCA was added to get 10% (w/v) final concentration as in the case of aliquot (b). All mixtures were incubated at -20°C overnight and precipitated protein pellets were collected by centrifugation (18,000 g, 15 min, 4°C). Pellets were washed with one volume of 100% ice cold acetone, allowed to air dry and suspended in appropriate volume of 2-DE rehydration buffer. Dissolved protein content was estimated by Bradford's method and then samples were subjected to 2-DE [20].

In-gel digestion of selected protein spots

Protocol of was followed. Gels were washed for 10 min. with 10% alcohol for two times. Protein spots of interest were excised (1 mm³ size) from 2D gels using spot picker and collected in 96 well PCR plate. Digestion of proteins and spotting of peptides on MALDI-TOF target plate was carried out using protein digester. Gel plugs were destained and dehydrated by washing three times (for 10 min) with 25 mM ABC-50% ACN (1:1). Dried gel plugs were treated with freshly prepared 10 mM DTT in 50 mM ABC at 56°C for 45 min. After incubation, DTT was replaced quickly by the same volume of freshly prepared 55 mM iodoacetamide in 50 mM ABC for 30 min. Gel plugs were again washed with 25 mM ABC and dehydrated with 100% ACN. Dried gel pieces were incubated for 12 hrs at 37°C with 25 mM ABC containing 0.02 µg/µl of mass spectrometry grade trypsin. The digestion reaction was stopped by adding 0.3% FA. Resulting peptides were extracted twice from the gel pieces using extraction buffer and transferred to a new 96 well PCR plate [21].

Bioinformation analysis

Bioinformatic analysis of identified proteins with unknown function was carried out using battery of different online servers and softwares.

Phylogenetic analysis

Orthologs of proteins from other species of mycobacteria and human were obtained from Kyoto Encyclopedia of Genes and Genomes (KEGG) (<http://www.genome.jp/kegg/>) by single-directional best-hit option (SBH) and same server was employed for MSA using ClustalW. We used the following combined set of 5 organisms: mtu (*M. tuberculosis*), mbo (*M. bovis*), mav (*M. avium*), mle (*M. leprae*) and hsa (*Homo sapiens*). Multiple alignments of protein sequences are important tools in studying sequences. Further evolutionary

relationships were established by Phylograms. Phylogram is a branching diagram (tree) assumed to be an estimate of a phylogeny, branch lengths are proportional to the amount of inferred evolutionary change [22].

Polymerase Chain Reaction (PCR) and Gel Extraction

Primers designed for cloning often give non specific bands due to incorporation of restriction sites. Thus for every pair of cloning primers optimum annealing temperature is to be determined. This was done with 25 μ l reactions with a temperature gradient PCR setup containing 1X Taq polymerase buffer, 200 μ M dNTPs, 1 μ M (each) primer and 2U of Taq polymerase. 2 μ l of template DNA (100 ng) from H37Rv was added to reaction mix. The thirty PCR amplification cycles carried out in iCycler Thermal Cycler were: denaturation at 94°C for 1 min, annealing at a temperature gradient from 52.0°C through 57.0°C (52.0, 53.0, 54.0, 55.0, 56.0, 57.0, °C) for 1 min and extension at 72°C for 2 min. Amplification was preceded by an initial denaturation at 95°C for 10 min, followed by final extension at 72°C for 10 min. Reaction was kept at 4°C until used. Amplified product was resolved on 1% agarose gel. Finally, amplification of the genes was performed at 54.0°C annealing temperature (optimum for both genes) for 1 min and extension at 72°C for 2 min. Amplified product was resolved on 1% agarose gel followed by gel extracted using MEGAquick-spin™ PCR & Agarose gel DNA extraction kit as per the manufacturer's protocol described earlier [23].

Preparation of Competent Cells of E.coli DH5 α & BL21

Competent cells were prepared by CaCl₂ method. E.coli strains DH5 α & BL21 were grown in 5 ml LB broth at 37°C for overnight. This culture was inoculated in 100 ml of fresh LB media and grown till OD₆₀₀ 0.4. Cells were centrifuged at 3000g for 10 min at 4°C and supernatant was discarded. Cell pellet was suspended in 10 ml of 0.1M CaCl₂ and kept on ice for 30 min, again centrifuged at 3000g for 10 min at 4°C and supernatant was discarded. Cells were resuspended in 1.5 ml of 0.1M CaCl₂ containing 40% glycerol by gentle pipetting. Prepared competent cells were kept in -80°C in 200 μ l aliquots [24,25,26].

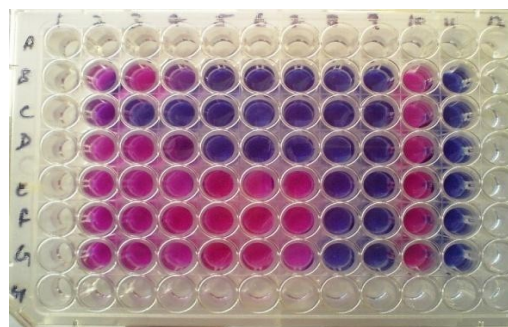
Results

Drug susceptibility profiles of M.tuberculosis isolates

In the present study three OFX mono- resistant and six total sensitive (to five first line drugs: RIF, INH, EMB, SM & PZA) M.tuberculosis clinical isolates were obtained from the Mycobacterial Repository Centre of our Institute. Drug susceptibility testing was performed for five first-line anti-TB drugs by conventional LJ proportion method.

Determination of OFX, CFX and MOX MICs by REMA platemethod

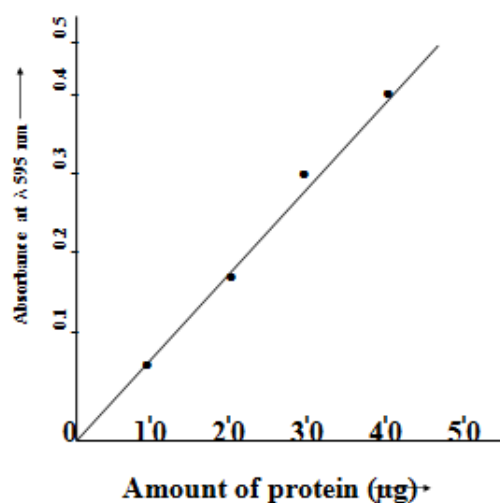
All M.tuberculosis isolates were tested for their MICs against OFX, CFX and MOX using REMA plate method. MICs were determined after addition of resazurin to the wells of microtiter plate. A change in colour of resazurin dye from blue to pink indicates reduction of resazurin and therefore bacterial growth. The MIC was defined as the lowest drug concentration that prevented this color change. Breakpoint drug concentrations defining drug resistance were determined for each drug.



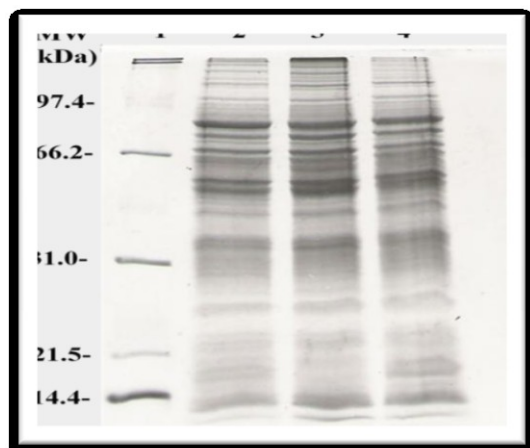
Photograph of resazurin microtiter assay plate method results. Pink wells indicate positive growth of M.tuberculosis while blue wells indicate inhibition of bacterial growth.

Protein estimation in whole cell lysate by Bradford assay

Protein concentrations in all samples were estimated by Bradford's method using BSA (1.0 mg/ml) as the standard, absorbance at 595nm was recorded and the protein concentration was determined by comparison to a standard curve (for BSA) as shown in Fig. 4.2. Protein concentrations were found to be in the range of 5.0-7.0 μ g/ μ l for M.tuberculosis susceptible and resistant isolates.



Amount of protein (µg) Standard curve of BSA for protein estimation by Bradford's method.



Gel electrophoresis profile of cell lysate proteins: Lane-1 Molecular mass marker, Lane-2 DKU-317 (OFX Induced), Lane-3 DKU-317 (CFX Induced), Lane-4 DKU-317 (Uninduced).

Bioinformatic analysis of all hypothetical proteins Identified proteins with unknown function were bioinformatically characterized by using different servers and software's such as BLASTP, InterProScan, Multiple Sequence Alignment and Molecular Docking. These are universal stress protein (Rv2623), uncharacterised protein (Rv1827), universal stress protein (Rv1636), 35 kDa protein (Rv2744c), conserved hypothetical protein (Rv2140c), putative CoA-transferase subunit alpha (Rv3551) and hypothetical protein (Rv0148).

Phylogenetic analysis

Orthologs of proteins from other species of mycobacteria and human were obtained from KEGG (<http://www.genome.jp/kegg/>) by single-directional best-hit option (SBH) and same server was employed for multiple sequence alignments (CLUSTALW). Multiple sequence alignment of mtu (*M. tuberculosis*) proteins was performed for the set of five organisms, mbo (*M. bovis*), maf (*M. africanum*), mav (*M. avium*), mle (*M. leprae*) and hsa (*Homo sapiens*). Results show that all proteins exhibited 100% homology (except Rv2623-55.3% homology) to their corresponding proteins in *M. bovis* and *M. africanum* which are members of tuberculosis complex. Rv2623 showed 55.3% with *M. africanum*, 56.3% with *M. avium*, 25.5% with *M. leprae* and 27.8% homology with human protein and Rv1827 exhibited 91.2% with *M. avium*, 87.6% with *M. leprae* and 29.7% homology with human. Rv1636 had 88.4% with *M. avium*, 89% with *M. leprae* and no homology with human protein. Rv2744c exhibited 88% homology with *M. avium*, 90.40% homology with PspA of *Mycobacterium liflandii* and 24.6% homology with myosin heavy chain of human and 26.6% homology with cell division protein of *M. leprae*. Rv2140c showed 83% with *M. avium*, 86.3% with *M. leprae* and 26.4% homology with peroxisome proliferator-activated receptor α of human. Rv3551 showed 87.2% with *M. avium*, 25.2% homology with

serine protease of *M. leprae* and 24.8% homology with 3-oxoacid CoA transferase 2. Rv0148 showed 87.6% homology with *M. avium*, but 50% & 33.5% homology with human and *M. lepreproteum*.

Multiple sequence alignment of the identified protein with defined set of organisms

	mb o (<i>M.</i> bovis)	Maf (<i>M.</i> africanum)	Mav (<i>M.</i> avium)	Mle (<i>M. leprae</i>)	Has (<i>Homo sapiens</i>)
Rv2623	100%	55.3%	56.3%	25.5%	27.8%
Rv1827	100%	100%	91.2%	87.6%	29.7%
Rv1636	100%	100%	88.4%	89.0%	-
Rv2744c	99.6%	99.3%	88.0%	26.6%	24.6%
Rv2140c	100%	100%	83.0%	86.3%	26.4%
Rv3551	99.7%	99.7%	87.2%	25.2%	24.8%
Rv0148	100%	100%	87.6%	33.5%	50.0%

InterProScan analysis

Motif and domain searches were made on EBI server (<http://www.ebi.ac.uk/Tools/InterProScan/>) employing InterProScan. InterProScan analysis of Rv2623 showed one Usp domain (# PF00582) which provides a signature for Adenine nucleotide alpha hydrolases activity (#SSF52402). Rv1827 showed one FHA domain (# PF00498). Rv1636 showed two UspA motifs from residues 10-148 and 162-293 (# PF00582), which provides a signature for Adenine nucleotide alpha hydrolases activity (#SSF52402). Rv2744c showed the presence of PspA domain with amino acid residues from 3-242 (PF04012). Rv2140c showed motif (#PF01161) from residues 17-175 characteristic motif of Phosphatidylethanolamine-binding protein and Raf kinase inhibitor-like protein. Rv3551 showed motif (#PF01144) from residues 6-233 characteristic motif which provides a signature for coenzyme A transferase. Rv0148 showed motifs (#PF00106) from residues 8-183 characteristic motif which provides a signature for short chain dehydrogenase.

Cloning of Hypothetical Proteins

Five identified proteins with unknown function (Rv1636, Rv1827, Rv2623, Rv0148 and Rv3551) were selected for further cloning and expression studies to find out its correlation with OFX, CFX & MOX mechanism of action and resistance.

PCR for Amplification of Rv1636, Rv1827, Rv2623, Rv0148 & Rv3551 Genes

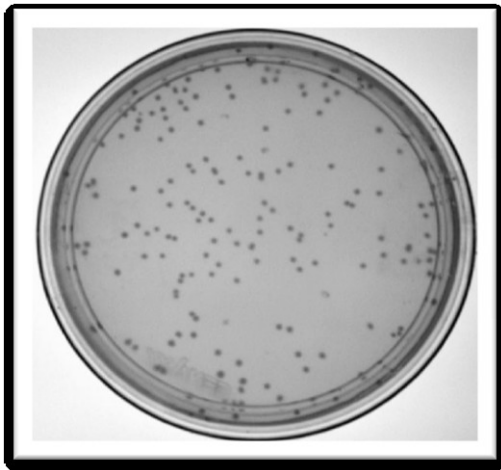
By using temperature gradient PCR setup, annealing temperature was optimized (54°C). Optimum annealing temperature at 54°C for 1 min (optimum for all five genes) was selected and PCR was performed. Amplified PCR product was resolved on 1% agarose gel followed by gel extraction.

Ligation and Transformation

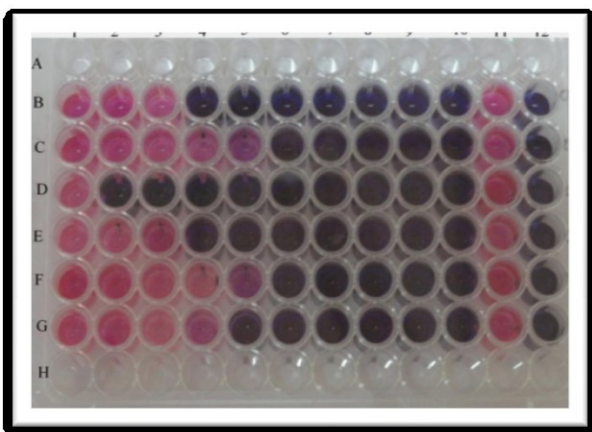
Ligation and transformation in E.coli DH5α & E.coli BL21 were performed as described in Methods section. After incubation at 37°C for 14 hrs colonies appeared on LB plates indicating successful transformation.

Isolation and Restriction Digestion of Recombinant pQE2 Plasmid

Plasmid DNA was isolated using DNA-Spin™ Plasmid DNA Purification kit and 1% agarose gel was used for analysis followed by gel extraction. Purified plasmids were restricted, analyzed on 1% agarose gel and fall outs of Rv1636 (~441bp), Rv1827 (~481bp), Rv2623 (~894) Rv0148 (~861 bp) and Rv3551 (~879 bp) were purified by gel extraction.

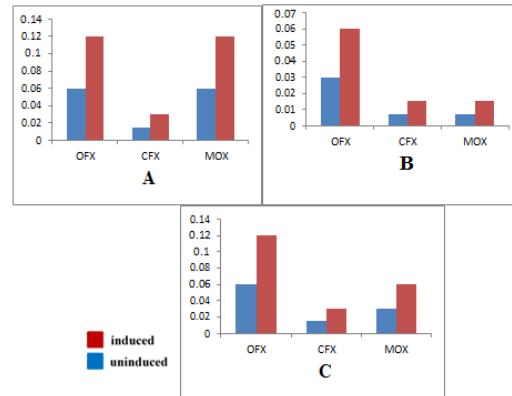


Petri plate with LB agar and 100µg/ml ampicillin showing transformation efficiency (1.5×10^2 CFU/ng) of DH5α/BL21 E.coli strain with pQE2 cloning vector with insert



REMA results for BL21 (DE3) having Recombinants after overnight incubation.

- Each plate containing
- RowB. Uninduced culture against OFX
- RowC. IPTG induced culture against OFX
- RowD. Uninduced culture against CFX
- RowE. IPTG induced culture against CFX
- RowF. Uninduced culture against MOX & RowG. IPTG induced culture against MOX



Graphs showing change in MIC of uninduced and IPTG induced cultures:

- (A). pQE2+Rv2623 construct in BL21 (E.coli),
- (B). pQE2+Rv1827 construct in BL21 (E.coli),
- (C). pQE2+Rv1636 construct in BL21 (E.coli).

Summary

Mycobacterium tuberculosis is the etiologic agent of tuberculosis (TB), a potentially fatal illness, which results in approximately 2 million deaths worldwide each year. Globally, emergence of drug resistance is a dangerous alarm. The increase in the incidence of MDR-TB and the emergence of XDR-TB presents tremendous challenges to the global efforts to battle against tuberculosis. Emergence of drug resistant strains indicates not only the need of search for new diagnostic markers, drugs or amendment in the second line treatment regimens, but also to explore the unknown mechanisms of resistance in M.tuberculosis for developing novel drug targets. Exploration of underlying unknown mechanisms responsible for resistance may unravel the leads for the development of newer therapeutics / markers/vaccines. Fluoroquinolones also have excellent in vitro and in vivo activity against M.tuberculosis and have proven to be among the most effective second line drugs used for the treatment of individuals infected with MDR-TB and patients experiencing severe adverse effects due to first-line drugs. Cumulative mechanisms associated with resistance to FQs are still fragmentary; the present study was designed to investigate the proteome analysis of drug-resistant and drug-susceptible M. tuberculosis isolates exposed to sub- MIC concentrations of FQs—ofloxacin (OFX), ciprofloxacin (CFX) and moxifloxacin (MOX), using proteomic and bioinformatic approaches.

Such information could be helpful for the development of newer diagnostics and therapeutic agents for better treatment.

Conclusions

In this report we employed comparative proteomic and bioinformatic approach to describe the whole cell proteome of FQs (OFX, CFX and MOX) induced and uninduced susceptible isolates and OFX mono-resistant *M.tuberculosis* isolates induced with OFX. In the first phase of the study with susceptible isolates we found eleven proteins overexpressed. Among these eleven proteins, only eight were with defined roles and three were with unknown functions. Bioinformatic analysis revealed the presence of conserved motifs and domains in the three hypothetical proteins. Molecular docking showed proper interaction of these drugs with hypothetical proteins (Rv2623, Rv1827 and Rv1636). It is presumed that overexpression of these proteins might be neutralized/inhibited/modulated by the drug molecule. When these genes (Rv2623, Rv1827 and Rv1636) were cloned and expressed in *E.coli* BL21 using pQE2 expression vector and susceptibility testing against FQs was carried out, two fold changes were observed in the MIC of cloned proteins, suggesting their probable roles in adaptation/survival during growth arrest and in maintaining cellular physiology of themycobacteria. In the second phase of the study with OFX mono-resistant isolates, six proteins (Rv0685, Rv0363c, Rv2744c, Rv3803c, Rv2534c and Rv2140c) were found to be upregulated in OFX mono-resistant isolates when compared with total sensitive isolates. Along with these six proteins, eight more proteins (Rv1475c, Rv0440, Rv2245, Rv1436, Rv3551, Rv0148, Rv2882c and Rv0733) were found to be upregulated in induced OFX mono-resistant isolates on comparing with uninduced ones. Bioinformatic analysis of the four proteins with unknown function (Rv2744c, Rv2140c, Rv3551 and Rv0148) revealed the presence of conserved motifs and domains in these hypothetical proteins. Molecular docking showed proper interaction of OFX with these proteins and suggested that these molecules might be overexpressed to inhibit/modulate/compensate the effect of the drug. Two fold changes were observed in the MIC of cloned proteins, suggesting their probable roles in conferring resistance. Another major finding of the study was the overexpression of various enzymes (fructose-bisphosphate aldolase, putative CoA-transferase subunit alpha, glyceraldehyde-3-phosphate dehydrogenase, KsA, adenylate kinase and aconitate hydratase) and EF-Tu, EF-P and Ribosome recycling factor, involved in major metabolic processes and translation machinery, crucial for the initial survival of the cells under prolonged adverse conditions. These preliminary findings need further validation and subsequently, exploitation for the development of novel diagnostic markers or newer therapeutic agents targeting directly to gene/protein responsible for drug resistance. This could open new windows of hope for mankind to fight

against the deadly disease of tuberculosis, especially multidrug resistant one.

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