Estimation of Efflux Mediated Multi-drug Resistance and its Correlation with Expression Levels of Two Major Efflux Pumps in mycobacteria


ABSTRACT

Multidrug resistance has been posing an increasing problem in the treatment of tuberculosis. Mutations in the genomic targets of drugs have been identified as the major mechanism behind this resistance. However, high degree of resistance in some isolates towards major drugs like rifampicin, isoniazid, ethambutol and streptomycin can not be explained solely on the basis of mutations. Besides this, certain other mechanisms like efflux pumps have also been considered as alternative mechanisms in the drug resistant isolates where there is no mutation and these mechanisms are specially important for drug resistance in non-tuberculous mycobacteria (NTM). In this study, we have estimated efflux pump mediated drug resistance in different mycobacterial species with the help of efflux pump inhibitors. All major anti-tuberculous drugs have been shown to be extruded by efflux pumps and the degree to which these drugs are extruded, vary in different mycobacterial species and isolates. The correlation of this resistance with functional activity of two major efflux pump genes pstB and Rv1258c was also assessed by reverse transcription PCR. Besides the significant role of these pumps observed, other efflux pumps, present in mycobacteria, may also be involved in drug resistance and need to be investigated.
INTRODUCTION

Mycobacterium has long been known to be an important genus among the major pathogenic organisms and tuberculosis caused by Mycobacterium tuberculosis is considered as largest killer among infectious diseases with high morbidity and mortality. Increasing multi-drug resistance towards anti-tuberculose drugs has focused medical researchers' attention to find solution to the phenomenon. Genomic mutations in the drug targets inside the bacterial cell have been identified as the major region behind this resistance. These mutations either alter the structure of the drug targets inside the bacterial cell, which make them unrecognizable by the drug or interfere with the normal drug action pathways and finally the targets. Although the presence of these mutations in drug resistant M. tuberculosis isolates has been reported in several studies, there is a section of isolates which do not possess these mutations despite being drug-resistant. Also, some of the isolates show exceptionally high degree of drug resistance which can not be explained solely on the basis of mutations. These facts have led to search for alternative mechanisms of drug resistance which is mediated by efflux pumps. These efflux pumps are proteins of plasma membrane which take part in the extrusion of certain substances like metabolites, excess ions and foreign toxic substances. Drugs are also being effluxed by these pumps being considered as foreign toxic substances. The organism can use these pumps as an alternative mechanism of drug resistance. A large number of efflux pumps have been identified and characterized in bacteria and found to be responsible for effluxing of majority of antibiotics. On the basis of bioenergetics and structural criteria these multidrug efflux pumps can be divided into two major classes. Secondary multidrug transporters utilize the trans-membrane electrochemical gradient of proton or sodium ion to efflux the drug from the cell. On the other hand, ABC (ATP Binding Cassette) type multidrug transporters use the free energy of ATP hydrolysis to pump drugs out of the cell.

Secondary multidrug transporters include Major Facilitator Superfamily (MFS), Resistance Nodulation Division family (RND), Small Multi-drug Resistance family (SMR) and Multidrug and Toxic compound Extrusion family (MATE). Since the discovery of LfrA, the first reported efflux pump in mycobacteria, a number of efflux pumps that confer resistance to one or several compounds have been described in the genus. These include EfpA, conferring resistance to various antimicrobials in M. tuberculosis, M. bovis and M. leprae, Tap, conferring resistance to aminoglycosides and tetracyclines in M.fortuitun and M.tuberculosis, mtp1 (PstB), conferring resistance to ciprofloxacin in M.smegmatis, Mmr, conferring resistance to erythromycin and ethidium bromide in M.tuberculosis, P-55 conferring resistance to aminoglycoside and tetracyclines in M.bovis, drdAB, conferring resistance to dauxorubicin and daunorubicin in
and other Mycobacterial Diseases, Agra as slants on Lowenstein-Jensen medium. The cultures were adapted in liquid Sauton's medium at 37°C. After obtaining sufficient growth these were scaled up to larger volumes.

Blocking experiments: Determination of efflux mediated drug resistance was done by growing the cultures in bottles containing increasing concentrations of individual anti-tuberculous drug with and without efflux pump inhibitors CCCP (Carbonyl cyanide chlorophenylhydrazone, Sigma) and Verapamil (Sigma). Bottles were incubated at 37°C. Readings were taken between 20-25 days after inoculation.

Drug induction and RNA isolation: Flasks with 50 ml Sauton's medium were inoculated with mycobacterial suspensions and incubated at 37°C with continuous shaking at 120 rpm. After achieving log phase on around 20-25 days, individual anti-tuberculous drugs were added at sub-inhibitory concentrations determined earlier in blocking experiments. Cultures were re-incubated for 16-18 hours more prior to RNA isolation under similar conditions. RNA was isolated by method earlier standardized with lysis in French Pressure Cell (Sim Aminko, USA) twice at 20,000 psi pressure and precipitation by absolute ethanol. Contaminating DNA was digested by treatment with 1U DNAase (Ambion) per 10 µl RNA sample. OD was taken at 260 and 280 nm to calculate yield and purity of RNA samples.

MATERIAL AND METHODS

Culture conditions: Well characterized mycobacterial cultures (drug sensitive as well as drug resistant) were obtained from Mycobacterial Repository Centre of National JALMA Institute for Leprosy and other Mycobacterial Diseases, Agra as slants on Lowenstein-Jensen medium. The cultures were adapted in liquid Sauton's medium at 37°C. After obtaining sufficient growth these were scaled up to larger volumes.

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RT-PCR: RT-PCR was done with RT-PCR kit (Bangalore Genei) according to the manufacturer’s protocol with primers for Phosphate specific ABC transporter gene pstB and MFS transporter gene Rv1258c. Reaction was carried out at 42°C for 1 hour. Primers for pstB were: (F) 5’GGTAAGTACCAACCCGCACGGC 3’, (R) 5’ CTCGTTGTAACGCATGGCGGC 3’ and for Rv1258c were (F) 5’ GGCCGCG GGTGATGCCGTCTCGAT 3’ (R) 5’ ATGCCGCAACCGTGCCGATCATC AAG 3’. PCR conditions for pstB amplification were initial denaturation at 94°C for 4 min then 25 cycles of 94°C for 1 min, 52°C for 30 sec and 72°C for 1 min followed by final extension at 72°C for 10 min and for Rv1258c amplification were initial denaturation at 94°C for 2 min then 30 cycles of 94°C for 1 min, 66°C for 1 min and 72°C for 1 min followed by final extension at 72°C for 5 min. Results were analyzed by quantity one software (BioRad, USA).

RESULTS

Results of blocking experiments were recorded on the basis of density by McFarland index and CFU count on LJ medium. Decrease in the minimum inhibitory concentration (MIC) of the isolates for a particular drug in the presence of efflux pump inhibitor was recorded which is a clear depiction of significant contribution of efflux pumps in conferring drug resistance.

The results of the blocking experiments of the isolates for individual anti-tuberculous drugs in the presence and absence of inhibitors are summarized in table1.

In the RT-PCR amplification, pstB was shown to be over-expressed in case of resistance to ethambutol, rifampicin and

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Species</th>
<th>Drug resistance profile (resistant to)</th>
<th>Inhibitor used</th>
<th>Drugs for which maximum reversal of resistance was found</th>
</tr>
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<tr>
<td>ICC 751</td>
<td><em>M. tuberculosis</em></td>
<td>RHESO</td>
<td>CCCP</td>
<td>EMB, RIF, OFL</td>
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<td></td>
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<td>Verapamil</td>
<td>STR, RIF</td>
</tr>
<tr>
<td>MH 28</td>
<td><em>M. tuberculosis</em></td>
<td>RHESO</td>
<td>CCCP</td>
<td>RIF, OFL</td>
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<td>TBDC 4</td>
<td><em>M. tuberculosis</em></td>
<td>RHESO</td>
<td>Verapamil</td>
<td>RIF, OFL EMB</td>
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<td>ICC 55</td>
<td><em>M. fortuitum</em></td>
<td>RHESO</td>
<td>CCCP</td>
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<td>M. smeg</td>
<td><em>M. smegmatis</em></td>
<td>RHES</td>
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<td>2 S</td>
<td><em>M. phlei</em></td>
<td>RHES</td>
<td>CCCP</td>
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<tr>
<td>ICC 264</td>
<td><em>M. flavescence</em></td>
<td>RHESO</td>
<td>CCCP</td>
<td>STR</td>
</tr>
<tr>
<td>ICC 280</td>
<td><em>M. avium</em></td>
<td>RHESO</td>
<td>CCCP</td>
<td>INH</td>
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rifampicin+isoniazid in *M. tuberculosis* (Fig 1). Ethambutol and rifampicin were also observed to be effluxed out in the blocking experiments. When tested in *M. smegmatis*, pstB shown to have hyper-
activity in case of resistance to streptomycin and rifampicin+isoniazid conditions (Fig 2). Its activity in *M. smegmatis* has also been reported in case of fluoroquinolones. When Rv1258c was studied in *M. tuberculosis* it showed virtually similar expression in all the drug induced conditions tested but higher than in case of drug free condition when analyzed over gel documentation system (Fig 3).

**DISCUSSION**

The role of efflux pumps in conferring drug resistance has long been recognized both in eukaryotes and prokaryotes. In mycobacteria, the association has been recognized since the discovery of LfrA in *M. smegmatis*. Being one of the major mechanisms of drug resistance in case of various types of cells and organisms, the need to study efflux pumps in mycobacteria has been strongly felt by researchers. A number of efflux pumps in mycobacteria have been identified and their role in drug resistance has been discussed. Most of the studies were limited to non-pathogenic organism *M. smegmatis*, although a few workers have demonstrated involvement of efflux pumps in drug resistance also in *M. tuberculosis complex*. Where in laboratory conditions, efflux pumps have been shown to be inhibited by certain compounds (verapamil, CCCP, reserpine etc), clinical trials have also proven therapeutic roles of these inhibitors in the case of eukaryotic cells in cancer patients. Despite interesting

![Fig 1: pstB amplification by RT-PCR from *M. tuberculosis* (ICC-751) grown in different drugs conditions. Lane 1: Ethambutol 6 g/ml, lane 2: Rifampicin 1 g/ml, lane 3: Mix Drug (Rifampicin 0.25 g/ml + Isoniazid 0.25 g/ml + Ofloxacin 0.5 g/ml), Lane 4: Drug free, lane 5: H₃Rv DNA and M: 100 bp DNA molecular weight marker.](image)
14 subfamilies of MFS pumps and several ABC transporters including drug efflux pumps. Open reading frames annotated as drug efflux pump genes in *M.tuberculosis* are available at Sanger Institute's website. Most of these genes have not been characterized yet and their functional activity has not been investigated thoroughly.

Outcome from these studies, more information about the role of these pumps is required and confirmation and re-assessment of the observations is needed to provide a definitive direction for revision of therapeutic regimens. Cole et al. in 1998 sequenced the complete genome of *M.tuberculosis* and identified 400 bp amplified regions for pstB and Rv1258c genes.
demonstrated experimentally, which needs to be investigated by other molecular approaches. We have therefore assessed the efflux pump mediated drug resistance to anti-TB drugs in pathogenic as well as non pathogenic species of mycobacteria. In this study although we have found reversal of resistance in the presence of efflux pump inhibitors for every drug tested, most significant efflux was observed for ofloxacin and rifampicin in *M. tuberculosis* isolates. However, in other species of mycobacteria, isoniazid and streptomycin were the major targets of efflux pumps. The present study provides the information about efflux pump mediated drug resistance in *M. phlei* and *M. flavescence* for the first time as there is no study giving this type of information in these species. Although the role of efflux pump in drug resistance has been described earlier in *M. avium* and *M. fortuitum*, the present study has added up newer information about this type of mechanism in these species. Reverse transcription PCR showed role of phosphate specific transporter PstB in effluxing ethambutol out of the cell in *M. tuberculosis*. When also tested in *M. smegmatis*, streptomycin was the major drug effluxed out by PstB. Rv1258c have shown virtually similar expression in all drug induced conditions in the given isolate but higher as compared to drug free condition. Our findings provide interesting information about the role of efflux pumps in mediating drug resistance in a wide spectrum of mycobacterial species, but more experiments to generate comprehensive information about all the efflux pumps working together (alone or in combination) in different mycobacterial species is still required. Although more studies are needed, the initial outcome of the present work has been found interesting and would be useful for making further progress on this aspect which is therapeutically important.

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REFERENCES


