Resistome analysis of *Mycobacterium tuberculosis*: Identification of aminoglycoside 2′-N-acetyltransferase (AAC) as co-target for drug designing

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Abstract:
The emergence of multidrug resistant tuberculosis (MDRTB) highlights the urgent need to understand the mechanisms of resistance to the drugs and to develop a new arena of therapeutics to treat the disease. Ethambutol, isoniazid, pyrazinamide, rifampicin are first line of drugs against TB, whereas aminoglycoside, polypeptides, fluoroquinolone, ethionamide are important second line of bactericidal drugs used to treat MDRTB, and resistance to one or both of these drugs are defining characteristic of extensively drug resistant TB. We retrieved 1,221 resistant genes from Antibiotic Resistance Gene Database (ARDB), which are responsible for resistance against first and second line antibiotics used in treatment of *Mycobacterium tuberculosis* infection. From network analysis of these resistance genes, 53 genes were found to be common. Phylogenetic analysis shows that more than 60% of these genes code for acetyltransferase. Acetyltransferases detoxify antibiotics by acetylation, this mechanism plays central role in antibiotic resistance. Seven acetyltransferase (AT-1 to AT-7) were selected from phylogenetic analysis. Structural alignment shows that these acetyltransferases share common ancestral core, which can be used as a template for structure based drug designing. From STRING analysis it is found that acetyltransferase interact with 10 different proteins and it shows that, all these interaction were specific to *M. tuberculosis*. These results have important implications in designing new therapeutic strategies with acetyltransferase as lead co-target to combat against MDR as well as Extreme drug resistant (XDR) tuberculosis.

Keywords: Antibiotic resistance, *Mycobacterium tuberculosis*, Acetyltransferase, Network analysis.

Abbreviations: AA-amino acid, AT-Acetyltransferase, AAC-Aminoglycoside 2′-N-acetyltransferase, XDR-Extreme drug-resistant, MDR-Multidrug-resistant, Mtb-Mycobacterium tuberculosis, TB-Tuberculosis.

Background:
Tuberculosis (TB), a bacterial origin infectious disease caused by obligate human pathogen *Mycobacterium tuberculosis* (Mtb). TB as a single infectious disease is responsible for the leading cause of deaths in developing as well as developed countries. It is estimated that annually 2 million people are dying due to this treatable disease. According to World Health Organization (WHO) reports for the year 2010, 8.8 million incident cases of TB were estimated globally. The highest number of deaths was in the African region. Without treatment against TB, mortality and morbidity are high. Despite the overwhelming research going on to understand the pathogenesis of *Mycobacterium tuberculosis*, increasing drug resistance in pathogen requires development of new therapeutic and preventive strategies [1].
Co-infection with HIV has given new dimension to the TB epidemics. It has been reported that 1.1 million deaths among HIV-negative cases of TB and an additional 0.35 million deaths among people who were HIV-positive occurred [2, 3]. Major setback to the Global TB eradication program is the rise of Multidrug Resistant (MDR) and Extreme Drug Resistant (XDR) mutants of *M. tuberculosis*, which are resistant to the first line and second line of anti-tuberculosis drugs. Drug resistance can be defined as the temporary or permanent capacity of organisms and their progeny to remain viable or to multiply in the presence of the concentration of the drug that would normally destroy or inhibit cell growth [4]. Multi-drug resistant tuberculosis (MDR-TB) is a disease caused by strains of *M. tuberculosis* that are at least resistant to treatment with isoniazid and rifampicin. Extensively drug-resistant tuberculosis (XDR-TB) refers to disease caused by multidrug-resistant strains that are also resistant to treatment with any fluoroquinolone and any of the injectable drugs used in treatment with second-line anti-tuberculosis drugs (aminoglycosides, capreomycin, and kanamycin) [4]. There are many factors like clinical, biological and socioeconomic which are responsible for the rise of drug resistance associated with *Mtb* infection [5, 6, 7].

The resistance acquired by pathogen may be due to plasmid, which carries different antibiotic resistance genes. The other MDR mechanisms are due to sequential accumulation of chromosomal mutations in different drug resistant genes that commonly occurs in case of MDR-TB and XDR-TB. Chromosomal mutations may be responsible for the different effect like reduced permeability, increased efflux, enzymatic inactivation, or alteration of drug target [8]. In light of this, it becomes necessary to search for the new targets to contain the TB epidemic globally. To counter the drug resistance in *Mtb*, global efforts are on to explore novel strategies for drug development and search for new therapeutic molecules as a drug target. Methods such as rotations of antibiotic combinations, improved medical surveillance to ensure proper patient compliance towards drug therapy are proving less useful compared to speed with which pathogen is becoming resistant to drugs. Identification of new targets that may be less prone to mutations, search for new chemical modulators for known molecular targets, use of virulence factors as targets and ‘phenotypic conversion’, which aims to inhibit the resistance mechanism employed by the bacterium [9, 10]. In this era of “Omics” where various databases are available, use of computational approaches to mine the possible therapeutic target seems much feasible requiring future experimental validation.

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**Figure 1:** Progression of experiments in this study: A flowchart illustrating the progression of experiments in this study. Different aspects indicated in this are Database construction, curation of the resistance proteins, identification of common genes by network analysis, Phyllogenetic analysis for homolog analysis, Protein-protein interaction analysis, and structural analysis.

**Figure 2:** Illustration of the gene network: Hexagonal node represents antibiotics (Pink in colour) and circular nodes (Yellow) correspond to the individual genes in the network while the edge (Grey) indicate interactions between them. Enlarged view of network show the common genes present in all antibiotics. This study includes construction and analysis of molecular interaction networks, which provides a powerful means to understand the complexity of biological systems and to reveal hidden relationships between drugs, genes, proteins, and diseases. In recent years, several computational methodologies have been developed to predict and develop models to understand the complexity of diseases like tuberculosis [11-15]. Analysis of genetic makeup will provide information about the crosstalk between different proteins, which can provide a new
way to identify a potential targets. Here, we use wide-scale network and phylogenetic analysis of genes and proteins association to discover possible new target to combat against MDR-TB and XDR-TB. The network analyses reported here further help in identification of genes, which are activated in response to resistance against antibiotics. The complete methodology for this analysis is represented as flowchart (Figure 1). The study also identify some protein that could be explored for their use as drug ‘co-targets’.

Methodology:

Data collection and Network analysis
Genes and Proteins encoding for the antibiotic resistance against first line antibiotics, like, aminoglycoside, ethambutol, isonazid, pyrazinamide, fluoroquinolones and rifampin were retrieved from the Antibiotic Resistance Gene Database [16] and collected in form of subset of main database. We analyzed and compared the genes for their screening and inclusion criteria of interactions. The interaction network for these genes were build and visualized by Cytoscape v2.8.2 software [17, 18]. In order to map the common genes in the interaction network, we used Entrez Gene ID as the unique identifier for genes.

Sequence analysis and Phylogenetic analysis
The protein sequences that are found to be common through network analysis from M. tuberculosis H37Rv and other Mycobacterium species were retrieved from NCBI (http://www.ncbi.nlm.nih.gov). Similarity in the selected sequences were evaluated by Blastp [19]. The multiple alignments and phylogenetic analysis of selected sequences were done using MEGAS software [20, 21]. Furthermore, analysis of the conserved motif in common 53 proteins aa sequence was performed by MEME online server, which use comparative analysis mode for finding conserved motifs [22-24].

Protein Interaction Network
A proteome-scale interaction network of proteins in M. tuberculosis with aminoglycoside 2’-N-aminoglycoside 2’-N-acetyltransferase AAC was derived from the STRING database [25, 26], using ‘High-confidence’ and ‘Medium-confidence’ data. Coexpression and Occurrence analysis for this protein was obtained from STRING database. Blastp analysis [27] against a human protein database was done to validate that this particular protein not share any homology with human proteins.

Homology modeling and structural comparison
Seven different Aminoglycoside 2’-N-acetyltransferase (AAC) (AT-1 to -7) from different clads were selected and amino acid sequence analysis was done by performing a multiple sequence alignment using ClustalX and also conserved sequences and motifs were identified using PSI-BLAST search [28, 29] and Pfam database [30]. Amino acid sequence of selected aminoglycoside 2’-N-acetyltransferase (AACs) of M. tuberculosis was aligned with bovine trypsin sequence (PDB ID: 1M4D; Aminoglycoside 2’-N-aminoglycoside 2’-N-acetyltransferase (AAC) from Mycobacterium tuberculosis in complex with coenzyme A and aminoglycoside substrates; Resolution=1.8A°). The 3D models were generated using the MODELLER package (version 9.10) [31]. All the models were energy minimized using a conjugate gradient algorithm and short MD simulations, as part of the MODELLER protocol in order to refine the side chain orientations. 50 models were generated for each sequence, which were rated according to the GA341 and DOPE scoring functions [32]. The structures were analyzed using Pymol software [33, 34] and superimposed using TM align server (http://zhanglab.ccmb.med.umich.edu/TM-align/) [35]. The models were also analyzed for oligomeric states using SCORER 2.0 program [36, 37]. The 3-D structures of predicted models were validated with the programs PROCHECK and ProSA analysis. These programs generate

Figure 3: Phylogenetic analysis of common genes: Phylogenetic tree comprises of 53 common genes which were identified by network analysis. Shaded region shows sequences which code for acetyltransferase activity.
Ramchandran plots of the amino acid residues in the allowed region and consider the overall G-factors to give scores for predicting model quality.

**Figure 4**: Illustration of the AAC protein interaction from STRING database: A) Network of protein-protein Interaction of AAC in *M. tuberculosis*; B) Heat map showing degree of Coexpression in AAC interacting proteins; C) Prediction of Co-occurrence of ACC (Rv262c) with two hypothetical proteins Rv263c and Rv264c respectively.

**Result**:

All antibiotics share common genetic makeup for resistance

Network analysis shows that selected 1221 resistant genes form 1220 interactions with six nodes, which represent each individual antibiotic (Figure 2). Fifty-three genes were found to be common among selected first line and second line antibiotics resistant genes, whereas aminoglycosides and Fluoroquinolones share 41 common resistance genes. These common 53 genes encode for mechanisms of destruction and detoxification of antibiotics. Out of common 53 genes, 28 codes for aminoglycoside N-acetyltransferase, this modifies aminoglycosides by acetylation. Remaining genes were encoded for a product like Class A beta-lactamase (Q5951; This Enzyme breaks the beta-lactam antibiotic ring open and deactivates the molecule's antibacterial properties), tetracycline Efflux pump (AA84282, YP_889433), and Sulfonamide-resistant dihydropteroate synthase (Q49184). Another major group of genes (21 genes) produces pentapeptide as functional
Acetyltransferases shows divergent evolution

Outcome of multiple alignments (Figure 3) gives that, 53 amino acid sequences of common genes from Mycobacterium tuberculosis and related species of M. tuberculosis shares 90% of sequence similarity. Phylogenetic analysis shows that out of these 53 genes, 28 genes which codes for Aminoglycoside 2'-N-acetyltransferase (AAC) activity cluster together. This acetyltransferase cluster is subdivided in 7 different clad, signifies for small sequence variation in related sequences. All results from multiple alignment and phylogenetic analysis show that acetyltransferases in Mycobacterium tuberculosis were evolved divergently from ancestral component.

All acetyltransferase shares a common ancestral core

Predicted models of aminoglycoside 2'-N-acetyltransferases show common structure despite of their sequence divergence. According to Ramchandran pot analysis using PROCHECK, all predicted models have 98% of aa in favored and allowed region (Figure 5A). SCORER 2.0 analysis shows that all protein were predicted to form dimer. Each structure is composed of two interconnected beta sheets and four alpha helices. Strands $\beta 1-4$ form relatively flat antiparallel $\beta$ sheet, whereas strands $\alpha 1$, $\alpha 2$, $\alpha 3$ lie against the flat surface of $\beta$ sheet, whereas strands $\beta 5-10$ and portion of $\beta 3$, $\beta 4$ forms the open barrel with mixed topology. Helix $\alpha 4$ lies against the outer surface of this barrel. This all-structural features were remained common for all selected acetyltransferase, which leads to conclusion that all seven acetyltransferase share common ancestral structural core.

Validation of acetyltransferase as co-target for Anti-tuberculosis drugs

PyMol and Accelrys Discovery Studio Visualizer 3.0 (http://accelrys.com/) were used for structural analysis. The mapping of a conserved motif obtained from MEME analysis, on predicted models of acetyltransferases (Supplementary Figure 1) shows that the binding pocket of this particular class of protein is highly conserved in Mtb and related species (Figure 5B). Blastp analysis shown that (AT) from M. tuberculosis were unrelated to any class of human protein Table 1 (see Supplementary material). This all features makes acetyltransferases as potential co-target for existing and new developing class of drugs.

Discussion:

Complexity in “Omics” of M. tuberculosis and also the emergence of MDR and XDR strains gives it potential to be one of most lethal infectious pathogen. Multidisciplinary and multifaceted approaches can serve as better mean to solve the complexity of this pathogen. During the course of time, different terms like drugome, reactome, and pocketome had

M. tuberculosis aminoglycoside 2'-N-acetyltransferase (AAC) shows association with Non-Housekeeping genes:

From STRING database, proteome-scale interaction network of proteins in M. tuberculosis H37Rv was derived Table 1 (see supplementary material). This database takes account of interactions from published literature describing experimentally studied interactions as well as those from genome analysis using several well established methods such as domain fusion, phylogenetic profiling and gene neighborhood concepts. This network comprises of different types of interactions such as Coexpression, co-existence and common neighborhood (or domain fusion) of query protein. In our study, we found that aminoglycoside 2'-N-acetyltransferase (AAC) form M. tuberculosis is interacting with 10 different proteins as summarized in (Figure 4). Out of these 10 proteins, 5 proteins MT3587, MT2804, MT027, mmpS5 and MT0276 codes for hypothetical proteins, whereas remaining proteins are also of specialized function. Prediction of Coexpression shows that only three proteins pra, MT0185 and mmpS5 are associated with each other with medium confidence value (0.4) and also this association is specific for M. tuberculosis H37Rv (Figure 4).

From the STRING database Neighborhood analysis we can predict that aminoglycoside 2'-N-acetyltransferase AAC (Rv0262c) is closely associated with two hypothetical proteins Rv0264c and Rv0263c. Blastp analysis against Homo sapiens protein database show that aminoglycoside 2'-N-acetyltransferase AAC doesn’t share any homology with human protein.

Figure 5: Structural comparison between acetyltransferase: Conservation in structure between aminoglycoside acetyltransferase in M. tuberculosis. Common region from all seven structures is highlighted as ancestral core, which is shown at the centre; B) Mapping of conserved region obtained from MEME analysis (Ball and Stick form) and central sphere signifies for predicted binding site.
Emergence of drug resistance in *M. tuberculosis* leads to put efforts aimed at identifying new potent broad-spectrum drugs/antibiotics, but along with this, it is necessary to take account of drug resistance mechanism and the way to overcome this. Development of drug targeting co-target like Aminoglycosides transferases will be effective in enhancing efficacy of existing anti mycobacterial regime and it will provide additional strength for newly developing drugs.

**Conclusion:**

Network analysis of different antibiotic resistance gene in *M. tuberculosis* has provided the platform for identifications of co-targets. Further structural characterization and analysis using different tools like structural superimposition and binding site predictions might be useful for targeting these co-targets. Furthermore, it is also useful to develop several inhibitor molecules against these co-targets in process of anti-tuberculosis drug development.

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Supplementary material:

Table 1: Blastp analysis of *M. tuberculosis* Aminoglycoside 2'-N-acetyltransferase against non-redundant human protein dataset

| Query id | Subject ids | % identity | % positives | Alignment length | Mismatches | gap opens | q. start | q. end | s. start | s. end | E value | Bit score |
|----------|-------------|------------|-------------|------------------|-------------|-----------|----------|--------|----------|--------|---------|-----------|
| sp|P0A5N0|A|gi|119621264|g|51.85|62.96|27|13|0|150|176|93|119|0.68|33.5|
| AC2_MYCTU | b|EAX00859.1| 59.09 | 63.64 | 22 | 9 | 0 | 125 | 146 | 170 | 191 | 6.5 | 29.6 |
| sp|P0A5N0|A|gi|21739519|em|29.03|54.84|62|37|3|86|140|110|171|8|29.6|
| AC2_MYCTU | b|CAD38801.1| 29.03 | 54.84 | 62 | 37 | 3 | 86 | 140 | 110 | 171 | 8 | 29.6 |

Blastp; Iteration: 0; Query: sp|P0A5N0|AAC2_MYCTU Aminoglycoside 2'-N-acetyltransferase OS=Mycobacterium tuberculosis GN=aac PE=1 SV=1; RID: HWWY7CMR01R; Database: nr; 3 hits found.

Supplementary Figure 1: Mapping of conserved motif of divergent *M. tuberculosis* Aminoglycoside 2'-N-acetyltransferase sequence on quaternary structure of Aminoglycoside 2'-N-acetyltransferase.