

# Current Status and Unusual Mechanism of Multi-resistance in *Mycobacterium tuberculosis*

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#### Abstract

TB is a deadly disease and MDR-TB is spreading even DOTS drug regime has rigorously maintained with at least ten new drugs like isoniazid, capreomycin, dapsone, linezolid, pyrazinamide, ethambutol and bedaguiline apart from traditional drugs like rifampicin, streptomycin, amikacin and clarithromycin. Rifampicin inhibits RNA polymerase and mutations in rpo gene codons give resistance. Streptomycin inhibits protein synthesis and mutation of ribosomal proteins and rRNA genes give resistance but no strAB or mphA1-9 mdr genes reported in Mycobacterium. Bedaquiline kills M. tuberculosis by inhibition of the membrane-bound F, F,-ATP synthase complex. Ciprofloxacin and ofloxacin were replaced by moxifloxacin that binds to DNA gyrase inhibiting DNA replication and/or transcription and mutations of gyrA at position 90 and 94 and gyrB at position 74, 88 and 91 give resistance. Kanamycin and amikacin inhibit protein synthesis and mutation of rrs gene gives resistance. However, aac2'-lc type acetylating enzymes have also been suggested for multi-resistance. Ethambutol interferes with the biosynthesis of arabinogalactan in the cell wall and embB gene mutation at position 306 gives resistance. Erm<sup>MT</sup> methyl transferase adds methyl group to 23S rRNA at A<sup>2058</sup> giving resistance to azithromycin and clarithromycin where capreomycin or viomycin peptide antibiotics may be effective drug. Ethionamide is a derivative of isonicotinic acid inhibits mycolic acid synthesis disrupting membrane function. Ethionamide resistance were linked due to mutations in etaA, ethA, ethR and inhA genes. Isoniazid is also a pro-drug and katG gene (S315T) mutation was reported for its resistance. Pyrazinamide after conversion to pyrazinoic acid disrupts membrane function inhibiting ATP synthesis and pncA or rpsA gene mutation likely gives resistance. Cycloserine is a peptidoglycan synthesis inhibitor competing D-Alanine ligase. We find beta-lactamase (BlaC) and penicillin binding protein (penA) as well as well studied emrB and qacB drug efflux proteins by genome wide search. But no 50-500 kb MDR plasmid carrying five or more mdr genes as found in most Enterobacteriaceae, have not sequenced in M. tuberculosis. We conclude that search for Mycobacterium plasmids must be accelerated pointing multi-resistance. Surely, phage therapy and gene medicines also have got momentum to overcome multi-resistance and antibiotics void.

**Keywords:** MDR-TB; DOT drugs; Ethambutol; Ethionamide; Bedaquiline; Dapsone; Mycolic acid; katG; blaC; penA; QacA; ermB

#### Introduction

TB is caused by Mycobacterium tuberculosis and have many related species like M. africanum, M. bovis,, M. chimaera, M. celatum, M. smegmatis, M. branderi, M. cansasii, M. abscessus, M. fortunum, M. orygis, M. mungi, M. kansasii, M. gordonae, M. xenopi, M. marinum and M. pinnipedii. It is a serious disease where lungs' severely deformation occurs and such patients have short life. M. ovium, M. leprae, M. colombiense, M. indicus pranii and M. interacellulare do not affect lung. Robert Koch's discovered the tubercle bacillus in 1882 and was a major event in the history of medicine to conquest the deadly disease which had plagued mankind for centuries of speculation as to the possible infectious nature of tuberculosis [1]. In the past (1850s) one person out of seven were died due to tuberculosis before the discovery of tuberculin test by Koch and TB vaccination protocol. The TB bacilli grow very slow but M. abscesseus, M. fortuitum, M. canariasense, M. septicum, M. chelonae, M. boenickel species grow fast [2]. India is a big country and prevalence of TB is also greater than any country. Theobald Smith described Mycobacterium bovis to cause animal TB first and a wide range of animal hosts have been identified and characterized as M. microti, M. pinnipedii, M. mungi, M. caprae, M. orygis and M. suricattae [3,4]. A very long term treatment (6 months) is required for complete eradication of Mycobacterium tuberculosis from body because the bacilli can invade the cells of tissue, muscle, lung, bone so deeply that drug effective concentration hardly reach to kill the bacteria easily (Figure 1).



Contrary to *Escherichia coli*, no MDR conjugative plasmid has sequenced in *Mycobacterium tuberculosis* and thus no conjugation

sequenced in *Mycobacterium tuberculosis* and thus no conjugation experiment has conducted to confirm acquisition of *mdr* genes from MDR conjugative *Enterobacteriaceae* [5,6]. Extensively drug-resistant

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TB (XDR TB) is a rare type of MDR TB that is resistant to isoniazid and rifampin, plus any fluoroquinolone (ciprofloxacin) and at least one of three injectable second-line drugs (i.e., amikacin, kanamycin, or capreomycin. TB bacilli cannot survive in water resources and it is strictly host specific. Sadly, it was referred by WHO as the disease of India, The Mycobacterium genus comprises more than 70 species, including the major human pathogens responsible for tuberculosis is Mycobacterium tuberculosis and Mycobacterium leprae whereas most related infections being caused by Mycobacterium abscessus, Mycobacterium chelonae, and Mycobacterium fortuitum [7]. I will discuss the Mycobacterium specific drugs, drug resistance genes and their mutations. Mycobacterium has difference in cell wall chemistry (Figure 2) limiting hundreds of β-lactam drugs for TB and leprosy treatment whereas discovery of BlaC beta-lactamase proved that penicillin's could not be a good drug for TB [8,9]. The cell wall consists of the hydrophobic mycolate layer and a peptidoglycan layer held together by a polysaccharide, arabinogalactan which is the drug target and also gives acid fast limiting Gram staining. Although no MDR plasmid has isolated from Mycobacterium, hundred Mycobacteriophages have been isolated and their role in multi-resistance have not focused. Mycobacterium genome is large (>6000, 000 bp) and codes for more than 60000 proteins and bacteriophages may help for genome diversity (Figure 2) [10-13].

#### Prevalence, Pathogenesis, Diagnosis and Vaccination

Koch's idea using tuberculin protein as vaccination failed but tuberculin injection produced rash was found to be effective diagnosis for TB was developed by Leon Calmette and Charles Mantoux, known as "tuberculinisation" or tuberculin skin test. Camille Guérin discovered M. bovis, a non-infectious to rabbits but immunogenic and vaccination is protective. It is now known, a hundred years later, that M. bovis BCG is a variant harbouring more than 12 DNA deletions with regard to M. tuberculosis. About 80% of TB affects the lungs, it being an air-borne disease spread by careless acts of sneezing, coughing and spitting in public. The germs can spread via the bloodstream to lodge in any part of the body and manifest it in those sites. TB affects even the bones, commonly those of the spine and known as Bone-TB. The discovery of streptomycin in 1943 by Albert Schatz and his PhD student Selman Waksman (won Nobel Prize in 1952), was the great discovery concerning tuberculosis horror. Rifampicin another anti-tuberculosis drug was discovered in 1965, marketed in Italy in 1968, and approved

Figure 2: Structure of complex *Mycobacterium* cell wall giving ideal

Figure 2: Structure of complex *Mycobacterium* cell wall giving ideal site for drug design: 1-outer lipids, 2-mycolic acid, 3-polysaccharides (arabinogalactan), 4-peptidoglycan, 5-plasma membrane, 6-lipoarabinomannan, 7-phosphatidylinositol mannoside.

in the United States in 1971. Thus, there should not be any TB cases as streptomycin and rifampicin therapy drastically reduced death and carriers. But that was not the case for long time. Today about 9 million TB cases worldwide and more than half a million women and children have died per year worldwide [14]. This has happened due to appearance of drug resistant Mycobacterium tuberculosis which has chromosomal mutations and MDR plasmids. Major mdr-tuberculosis burden was found in the three big countries Russia (>46000 cases), China (>61000 cases) and India (>66000 cases) [15]. It mostly affects the lung tissues but other organ may also be affected. Among the diagnosis X-ray of the lung shows the heavy dense affected spot indicating progress of the disease. But such patients if not responded to the first line DOTS drugs, it is very hard to survive and usually die within months. Over the next 35 years, multidrug-resistant tuberculosis will kill 75 million people and could cost the global economy a cumulative \$16.7 trillion-the equivalent of the European Union's annual output, a UK parliamentary group said recently citing WHO data. The spread of TB cases in India has shown in Figure 3. Green areas are heavily affected in India but even it is the disease of the tropics, it has located worldwide. In 2016, an estimated 6.2% of people with MDR-TB had extensively drug resistant TB (XDR-TB) with as less as 30% therapeutic success (Figure 3) [16,17].

The proper diagnosis is important for any disease to know when the disease has started and how it is progressing in the population. Microbiology has started with pioneering work of Anton Von Leeuwenhoek, Louis Pasteur and Robert Koch (1680-1860). There are no evidences how pathogens exist in host making disease until advancement of molecular biology techniques in 1960s. The vast majority of studies detecting M. tuberculosis DNA sequences have used bone as the material whereas a few works used lung tissue. The oldest evidence for human tuberculosis was found in a Neolithic infant and woman in a 9000 years old settlement in the Eastern Mediterranean [18]. Typical clinical signs of tuberculosis reported earlier by a Roman physician (500 BC) with typical coughing associated with fever during the night with sweats. The military tuberculosis, as described by Thomas Willis 1660s seemed to be more frequent at that time than it is now, probably because it was the era before bacillus Calmette-Guérin (BCG) vaccination. Jean-Antoine Villemin in 1870s first demonstrated the transmissibility of tuberculosis.



In Mantoux screening test (tuberculin sensitivity test), a purified

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Figure 3: Prevalence of TB in Indian States. Green is >70% TB prevalence (Orisha, Jharkhand, Uttar Predesh) and yellow is >50% TB prevalence (Madha Pradesh, West Bengal, Assam, Tamil Nadu) and pink is >30% prevalence (Jammu Kasmir and Rajstan) and red is <30% TB prevalence which area is very small in India.

tuberculin protein derivative is injected into hand which shows red hypersensitivity scar indicating the positive tuberculosis. Tuberculin is a glycerol extract produced from TB bacilli culture filtrate. The tine test is a multiple-puncture tuberculin skin test used to aid in the medical diagnosis of tuberculosis and similar to the Heaf test, although the Mantoux test is usually used worldwide. However, X-ray of chest was important to see the scar and retractile nodules in the lungs showed in Figure 4. WHO classifies TB diagnosis into bacteriological confirmed TB or clinically diagnosed TB by WHO [19]. A posterior-anterior chest radiograph is used to detect chest abnormalities. Lesions may appear anywhere in the lungs and may differ in size, shape, density and cavity (Figure 4).

We see, Orissa, Andhra Pradesh, Uttar Pradesh and Gujarat have worse hit followed by Madhya Pradesh, West Bengal, Assam, Himachal Pradesh and Kerala. Jammu Kashmir, Maharashtra and Rajasthan states have less TB spread. Mycobacterium tuberculosis could be obtained from sputum or blood in blood-agar media in bacteriological test. The presence of acid-fast-bacilli on a sputum smear or other specimen often indicates TB disease but it does not confirm a diagnosis of TB because some acid-fast-bacilli are not M. tuberculosis. In molecular biology test, DNA was extracted from sputum or blood and PCR reaction was done using TB specific primers following agarose gel electrophoresis (NAAT). Extended of such studies could be restriction enzyme digest and Sanger di-deoxy DNA sequencing to confirm the gene sequence [20]. On September 30, 1999, the Food and Drug Administration approved a reformulated Amplified Mycobacterium Tuberculosis Direct Test (AMTDT) (Gen-Probe<sup>\*</sup>, San Diego, California) for detection of Mycobacterium tuberculosis in acid-fast bacilli smear-positive and smear-negative respiratory specimens from patients suspected of having tuberculosis [21].

### Materials and Methods

*Mycobacterium* is hard to grow quickly from host site. Egg-based Lowenstein-Jensen medium (LJ), American Trudeau Society medium (ATS), Stonebrink medium and agar-based Middlebrook 7H10 (7H10) were used for *Mycobacterium* growth with 80-90% successful. Ziehl-Neelsen stain and auramine phenol stain were used to identify acid-fast bacilli [22].

Importantly, DNA primers TB-1A, 5'-GAA CAA TCC GGA GTT GAC AA-3' and TB1B, 5'-AGC ACG CTG TCA ATC ATG TA-3') are used to amplify enough DNA for gel or DNA sequencing to confirm the



Figure 4: X-ray picture of chest showing patches of tuberculosis colony in the lung.

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presence of dormitory TB in patient refractive to X-ray test and blood culture test. However, may primers used in NAAT test are patented. In other methods, the sensitivity of the MTB/RIF RUO assay for smear-negative specimens was 60% for pulmonary and 75% for extrapulmonary specimens while the IS6110 LDT sensitivities was 40% and 0% respectively [21]. IS6110 encoded transposase gene (TnpA) is unique to *Mycobacterium tuberculosis* complex.

PCR amplification of the *mgtC* virulence gene using specific primers located approximately 80 bp upstream and downstream the *mgtC* gene may be useful for diagnostic. The primers are: mgtC-F (5'-CGC CTA GGC TCA AAC TGC TG-3') and mgtC-R (5'-CAA TAC CCG GCG GAT CTA CC-3'). The forward primer is located at nt. 505859 in H54 strain and at nt. 2033986 of CAS strain of *M. tuberculosis* (accession nos. CP019610 and CP028428 respectively). PCR mix was used with 10 ng of chromosomal DNA and each primer at 200 nM in a total volume of 50 µl with 0.2 mM dXTPs and 1.5 mM MgCl<sub>2</sub>. The reactions were initiated with a 5 mins denaturation at 95°C and primer extension was then carried out for 40 cycles as follows: denaturation for 30 sec at 95°C, annealing for 30 sec at 50°C, a 1 min extension at 72°C, and finally a 5 mins extension at 72°C. PCR products were visualized by using 1% agarose gel electrophoresis and were purified by using a NucleoSpin extract II kit followed by sequencing [23,24].

Interferon-y release assays (IGRA) are medical tests used in the diagnosis of some infectious diseases, especially tuberculosis. Interferon- $\gamma$  (IFN- $\gamma$ ) release assays rely on the fact that T-lymphocytes will release IFN-y when exposed to specific antigens. QuantiFERON-TB Gold which quantitates the amount of IFN-y produced in response to the ESAT-6 and CFP-10 antigens from Mycobacterium tuberculosis. The T-SPOT.TB, a form of ELISPOT determines the total number of individual T cells expressing IFN-y. Microscopy sputum test and DST for mdr-TB diagnosis have been introduced and modern detection method like GeneXpert has been standardized. 5 high MDR-TB countries, Ethiopia, Myanmar, Kazakhstan, Pakistan & Vietnam have treatment success > 70%. India lunched National TB Institute in 1959 for active research on TB diagnosis and drug discovery according to suggestion originated by ICMR survey in 1955-58 [25]. In 1962 NTP programmed started and 1993-1997 DOTS therapy (RNTCP: Revised National TB Control Programme) in effects every districts with 400000 centres nationwide at free of cost and 27 million TB suspected peoples were tested with 2.46 million TB positives identified and 87% of whom cured by DOTS (Directly Observed Treatment with Political commitment through Diagnosis by microscopy and Adequate supply of free drugs with Accountability) and also saved 80b\$ annually. India lunched in 2007 DOTSplus (PMDT) to cure mdr-TB.

## Results

#### MDR TB statistics and DOTs programme

An estimated 273,000 (95% confidence limits, 185,000 and 414,000) new cases of MDR TB occurred worldwide in 2000, 3.2% of all new TB cases [26]. In Global Tuberculosis Report 2017, India, China and other few countries were reported maximum MDR-TB incidence and mortality [27]. In India total 1936158 cases of TB were reported and TB + HIV peoples were maximum than only TB indicating huge spread of tuberculosis in India is due to unprotected sex of low income workers with infected sex partners. Multidrug-resistant was reported in India (147/1000), China (73/1000), Russia (63/1000) and Indonesia (32/1000) although death rate is much less in Russia indicating Russian are getting better treatment with costly new drugs [28]. Many middle African countries seem to have less MDR-TB and such low incidence

also reported in many highly populated South Asian small countries like Thailand (4.7/1000). It is unclear whether, remote villages were poorly screened in such countries. *Mycobacterium tuberculosis* MDR strains were detected worldwide with constant increasing trend. It detected in Indonesia [29], China [30,31], Mexico [2] and Africa [32].

TB Datasheets indicated that India was the worse country for TB incidence. In Table-1, we showed the 2017 Report and after India, African countries like Congo and Nigeria, then China and Bangladesh where major incidence of TB cases were reported.

# Old drugs streptomycin and rifampicin were wonderful for TB control but now useless

Rifampicin is made from the fermentation of soil bacterium Amycolatopsis rifamycinica and streptomycin by Streptomyces griseous [33-35]. IUPAC name of streptomycin 2-[(1R,2R,3S,4R,5R,6S)-3-(diaminomethylideneamino)-4is [(2R,3R,4R,5S)-3[(2S,3S,4S,5R,6S)-4,5-dihydroxy-6-(hydroxymethyl)-3(methylamino)oxan-2-yl] oxy-4-formyl-4-hydroxy-5-methyloxolan-2-yl] oxy-2,5,6-trihydroxycyclohexyl] guanidine. IUPAC name of rifampicin is (7S,9E,11S,12R,13S,14R,15R,16R,17S,18S,19E,2 1Z)-2,15,17,27,29-Pentahydroxy-11-methoxy-3,7,12,14,16,18,22heptamethyl-26-{(E)-[(4-methyl-1-piperazinyl)imino] methyl}-[23.3.1.14,7.05,28] 6,23-dioxo-8,30-dioxa-24-azatetracyclo triaconta-1(28),2,4,9,19,21,25 (29), 26-octaen-13-yl acetate. The structures of streptomycin and rifampicin were demonstrated in Figure 5.

Streptomycin resistance has occurred by mutation in 16S rRNA as well as ribosomal proteins [36]. Paromomycin and Levofloxacin are promise drugs now under clinical trials against streptomycin and

Country	Country MDR-TB Death/1000 infected		MDR-TB Death/1000 infected
India	423	Korea	11
China	50	Myanmar	25
Indonesia	110	Russia	12
Nigeria	115	South Africa	23
Congo	53	Thailand	8.6
Pakistan	44	Vietnam	13
Kenya	29	Zambia	4.8
Bangladesh	66	Congo	3.1
Philippines	22	Zimbabwe	1.2
Ethiopia	26	Namibia	0.75

 Table 1: Incidence of MDR-TB death in different countries as reported in Global

 Tuberculosis Report, 2017 (Higher rate of MDR-TB in India than any other country).



Figure 5: Structure of potent TB drugs Streptomycin and Refampicin which are now useless.

rifampicin resistant *Mycobacterium tuberculosis* (Clinical Trials ids. NCT00004444 and NCT00000796).

Mutations associated with streptomycin resistance in Mycobacterium tuberculosis have been identified in the rpsL and rrs genes, which encode the ribosomal protein S12 and 16S rRNA [37] respectively (Figure 6). Streptomycin inhibits protein synthesis by disrupting the relationship between these components to form functional ribosome with mRNA, aminoacyl-tRNAs and peptidyl transferase, inhibiting translation of mRNA and protein synthesis in bacteria. The 16S rRNA 530 loop region is part of the aminoacyl-tRNA binding site (A-site) and is involved in the decoding process where residues 526-CCG-524 and 505-GGC-507 plays a crucial role in translation. Thus little changes of this structural assembly generate resistance due to low affinity for streptomycin binding. Among the mutations reported in rpsL, a substitution in codon 43 from lysine to arginine has been the most commonly reported. This mutation produces high-level resistance to streptomycin [35-38]. There remain an important percentage of strains resistant to streptomycin that lack mutations in either of these two genes, suggesting additional mechanisms of resistance [39]. In 16S rRNA (rrs gene, the most common mutations occur around nucleotides 530, 915, 1400 and 1401 (Figure 6) [36,40]. StrA and StrB enzymes phosphorylate streptomycin and such genes are abundant in other MDR Enterobacteriace bacteria but there is no report of such genes in any plasmid of Mycobacterium. AadA1 gene coding for adenyl transferases that adenylate streptomycin has been well described as drug resistance marker as adenylated-streotomycin could not able to bind ribosome. Both *aad* and *aph* genes were found rare in Mycobacterium suggesting no MDR plasmid has discovered yet [16]. Many drugs have used but the TB cases are increasing due to multiresistance. But MDR-TB is less as compare to other bacterial diseases caused by many Enterobacteriaceae (E. coli, S. enterica, K. pneumoniae and A. baumannii). It appeared Mycobacterium spp. is very much host oriented and cannot survive in open water like river and sea limiting its conjugation with abundant MDR Escherichia coli [41]. So, we see poor correlations in plasmid-mediated mdr genes like blaNDM1, blaKPC, blaOXA, blaCTX-M, aacA1, aacC1, aph, cat, tet, sul1, dhfr, arr, mex, mcr, acr, norA, macAB, penA and tetM. Such discrepancy was reported in Neiserria gonorrhoeae recently by me (Figure 6) [42,43].

Rifampicin is a rifamycin derivative introduced in 1972 as an antituberculosis agent. It is one of the most effective anti-TB antibiotics and together with isoniazid constitutes the basis of the multidrug treatment regimen for TB [34]. The mode of action of rifampicin in *M. tuberculosis* is by binding to the  $\beta$ -subunit of the RNA polymerase, inhibiting the elongation of messenger RNA [44]. The majority of rifampicin-resistant clinical isolates of *M. tuberculosis* harbour mutations in the *rpoB* gene that codes for the  $\beta$ -subunit of the RNA polymerase. Mutant enzyme is refractive to bind rifampicin to inhibit RNA synthesizing activity. In about 96% of *M. tuberculosis* isolates resistant to rifampicin, hot-spot mutations were reported spanning codons 507-533 of the *rpoB* gene.

07010010	2261504	# 01 01 01 01 00 00 00 00 1 00 00 1 00 00
CB013613	3361224	TGASAGALTGCCGGGGTCAACTCGGAGGAGGTGGGGGATGACGTCATGCCGC 3361655
MG995516	1143	TGAGAGACTGOCGGGGTCAMCTCGGAGGAAGGTGGGGATGACGTCAAGTCATGCCCC 1202
CP019613	3361834	TTGTACACACOGCOCGTCACGTCATGAAAGTCGGTAACACOCGAAGCCAGTGGCCTAAOC 3361893
MG995509	1383	TTGTACACACOGCOCGTCRCGTCATGAAAGTCGGTAACACOCGAAGCCAGTGGCCTAACC 1442
	Dotocti	an of 100 point and a set of the la 14 sector is to be at a structure to be a secular
-igure 6:	Delecti	ion of 165 rRINA gene mutation in <i>Mycobacterium tuberculos</i>
M. tuberc	ulosis s	trains UKR40 and UKR32 16S ribosomal RNA gene (accessi
<i>Igure 6:</i> <i>M. tuberc</i> nos. MG9	ulosis s 995516	trains UKR40 and UKR32 16S ribosomal RNA gene (accessi and MG995509) were aligned with <i>M. tuberculosis</i> strain H1
M. tuberc nos. MG9 16S rRN	ulosis s 95516 A gene	trains UKR40 and UKR32 16S ribosomal RNA gene (accessi and MG995509) were aligned with <i>M. tuberculosis</i> strain H1 (accession no. CP019613 nt. 3360454-3361988). Mutati
M. tuberc nos. MG9 16S rRN egions w	eulosis s 995516 A gene vere only	trains UKR40 and UKR32 16S ribosomal RNA gene (access and MG995509) were aligned with <i>M. tuberculosis</i> strain H <sup>-</sup> (accession no. CP019613 nt. 3360454-3361988). Mutat y shown here and red nucleotide was mutated, M=A or C; R



However, mutations in codons 516, 526 and 531 are the most common with rifampicin resistance in the majority of studies [45]. MarRAB locus is involved in rifampicin resistance in *M. Smegmatis* [46]. Using the hot spot regions of katG and -15 region inhA, potentially to identify 89% of isoniazid resistance strains and the new assay which targets the rpoB516/526/531, katG315, gyrA94/91/90 and rrs1401 regions have the ability to distinguish 100% for rifampicin and isoniazid resistance strains (Figure 7) [47,48].

Thus all other drugs were tested for TB cure. Sadly, penicillin drugs were not very successfully used for TB management likely due to altered cell wall chemistry of the bacilli. An (E)-4-oxo-2-crotonamide derivative (IIIA16) was found to inhibit MDR-TB isolates with a MIC 0.005-0.48  $\mu$ g/ml [49] and new chemicals asperversiamides A-C could be interesting inhibiting *M. marinum* [50]. Recently Tükenmez et al. has disclosed tetrahydropyridine derivative as a new EPI against MDR-TB [51].

# Nobel TB-drugs and mechanisms of multi-drug resistance tuberculosis

Isoniazid was introduced in 1952 as an anti-TB agent and it remains, together with rifampicin, as the basis for the treatment of the disease. Isoniazid is only active against metabolically-active replicating bacilli. Isoniazid is a pro-drug that requires activation by the catalase/ peroxidase enzyme KatG, encoded by the katG gene, to exert its effect. Isoniazid acts by inhibiting the synthesis of mycolic acids through the NADH-dependent enoyl-acyl carrier protein (ACP)-reductase, encoded by inhA. Indeed, numerous studies have found mutations in these two genes as the most commonly associated with isoniazid resistance. Among these, the most prevalent gene mutation has been identified as S315T in katG resulting in an isoniazid product deficient in forming the isoniazid-NAD adduct needed to exert its antimicrobial activity. This mutation has been consistently associated with high-level resistance (MIC >1 µg/mL) to isoniazid and occurs more frequently in MDR strains and compensatory mutations have also determined [52]. The second most common mutation occurs in the promoter region of inhA causing an over expression of InhA or less frequently, a mutation in its active site, which decreases its affinity for the isoniazid-NAD adduct. The most prevalent mutation found is at position -15C/T and is more commonly associated with low level resistance to isoniazid (MIC <1 µg/mL). Mutations in the *katG* gene in MDR-TB account for 40-90% of INH-resistant strains. A recent study has also found that a silent mutation in mabA conferred isoniazid resistance through up-regulation of *inhA* in *M. tuberculosis* (Figure 8) [53].

Ethambutol was first introduced in the treatment of TB in 1966 and is part of the current first-line regimen to treat the disease. Ethambutol is bacteriostatic against multiplying bacilli interfering with the biosynthesis of arabinogalactan in the cell wall. In *M. tuberculosis*, the genes *embCAB*, organized as an operon, code for arabinosyl transferase, which is involved in the synthesis of arabinogalactan, producing the accumulation of the intermediate D-arabinofuranosyl-P-decaprenol. The recognized mechanism of resistance to ethambutol has been linked to mutations in the gene *embB* with mutations at position *embB306* as the most prevalent in most of the studies performed [54]. The same authors have more recently reported that mutations in the decaprenyl phosphoryl- $\beta$ -D-arabinose (DPA) biosynthetic and utilization pathway genes, Rv3806c and Rv3792, together with mutations in *embB* and *embC* accumulate, giving rise to a range of MICs of ethambutol depending on mutation type and number (Figure 9).

Pyrazinamide was introduced into TB treatment in the early 1950s and constitutes now part of the standard first-line regimen to treat the disease [55]. Pyrazinamide is an analogue of nicotinamide and its introduction allowed reducing the length of treatment to six months. It has the characteristic of inhibiting semi-dormant bacilli residing in acidic environments such as found in the TB lesions. Pyrazinamide is also a pro-drug that needs to be converted to its active form, pyrazinoic acid, by the enzyme pyrazinamidase/nicotinamidase coded by the pncA gene [56,57]. The proposed mechanism of action of pyrazinamide involves conversion of pyrazinamide to pyrazinoic acid which disrupts the bacterial membrane potential inhibiting membrane transport. Pyrazinamide would enter the bacterial cell by passive diffusion and after conversion to pyrazinoic acid. Under acid conditions, the acidified pyrazinoic acid would be reabsorbed into the cell and accumulated inside, due to an inefficient efflux pump, resulting in cellular damage. One study has also found that pyrazinoic acid and its n-propyl ester can inhibit the fatty acid synthase type I in replicating M. tuberculosis bacilli. Over expression of rpsA conferred increased resistance to pyrazinamide and pyrazinoic acid was confirmed to be bound to rpsA and likely a second target of pyrazinoic acid [58]. Mutations in the gene pncA remain as the most common finding in pyrazinamide resistant strains. These mutations, however, are scattered throughout the gene but most occur in a 561-bp region in the open reading frame or in an 82-bp region of its putative promoter. Based on the current evidence, the contribution of mutations in rpsA to pyrazinamide resistance



#### remains limited (Figure 10).

Fluoroquinolones are currently in use as second-line drugs in the treatment of MDR-TB. Both ciprofloxacin and ofloxacin are synthetic derivatives of the parent compound nalidixic acid, discovered as a byproduct of the antimalarial chloroquine. Newer-generation quinolones such as moxifloxacin and gatifloxacin are being evaluated in clinical trials and proposed as first-line antibiotics of short term treatment in TB. The mode of action of fluoroquinolones is by inhibiting the DNA gyrase and topoisomerase IV, two critical enzymes for bacterial viability. These proteins are encoded by the genes gyrA, gyrB, parC and parE, respectively. DNA gyrase is a tetramer formed by two  $\alpha$  and  $\beta$  subunits, coded by gyrA and gyrB, respectively, which catalyzes the supercoiling of DNA from relaxed ccc-DNA. The most frequent gyrA and gyrB mutations found are at position 90 and 94 of gyrA but mutations at position 74, 88 and 91 have also been reported [59]. Cross-resistance is assumed to occur between fluoroquinolones although isolated reports have acknowledged the presence of strains resistant to gatifloxacin and moxifloxacin that were still susceptible to ofloxacin. Hybrid fluoroquilone-flavonoid may be effective to curve XDR-TB [60].

Kanamycin, capreomycin, amikacin and viomycin antibiotics have the same mechanism of action by inhibiting the protein synthesis but



**Figure 9:** Mutational analysis of *embB* gene (1098aa) that gives ethambutol resistance in *M. tuberculosis*. Part of the align was shown here with I306M, G406A/C and A643T mutations. In *M. tuberculosis* XRD strain (resistant to rifampicin, ethambutol, isoniazid and streptomycin) has NH<sub>2</sub>-terminal 28 aa truncation (protein id. AEJ52310). R1002H mutation was evident in protein id. AJF45922.



while kanamycin and amikacin are aminoglycosides but capreomycin and viomycin are cyclic peptide antibiotics. All four are second-line drugs used in the management of MDR-TB. Kanamycin and amikacin inhibit protein synthesis by alteration at the level of 16S rRNA and ribosome. The most common mutations found are at position 1400 and 1401 of the rrs gene, conferring high-level resistance to kanamycin and amikacin. Capreomycin and viomycin, on the other hand, have a similar structure and bind at the same site in the ribosome, at the interface of the small and large subunits [46]. Mutations in the *tlyA* gene have also been associated with resistance to capreomycin and viomycin. TlyA is an rRNA methyltransferase specific for 2'-O-methylation of ribose in rRNA [61]. Macrolides are more frequently recommended for the treatment of other mycobacterial infections due to their limited activity against M. tuberculosis. Intrinsic resistance to macrolides has been attributed to low cell wall permeability and the expression of emr37, a gene that codifies for a methylase at a specific site in the 23S rRNA, blocking the binding of the antibiotic [39,62].

The mode of action of linezolid is by inhibition of an early step in the synthesis of proteins, binding to the 50S ribosomal subunit. A more recent study using next-generation sequencing has also found the mutation T460C in *rplC*, encoding the 50S ribosomal L3 protein, in in vitro-selected mutants and clinical isolates of M. tuberculosis resistant to linezolid. Previous studies have also found evidence of the possible involvement of efflux pumps in the resistance of M. tuberculosis to linezolid. On December 28, 2012, the U.S. Food and Drug Administration approved Bedaquiline for mdr-TB. Para-Amino Salicylic Acid (PAS) was one of the first anti-tuberculosis drugs used in the treatment of the disease, together with isoniazid and streptomycin. It has been proposed that being an analogue of para-amino benzoic acid, it must compete with it for di-hydropteroate synthase, interfering in the process of folate synthesis. A study using transposon mutagenesis identified mutations in the thyA gene associated with resistance to PAS that were also detected in clinical isolates resistant to PAS. A recent study has also identified various miss-sense mutations in folC encoding dihydrofolate synthase that conferred resistance to PAS in laboratory isolates of M. tuberculosis.

Ethionamide is a derivative of isonicotinic acid structurally similar to



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isoniazid. It is also a pro-drug requiring activation by a monooxygenase encoded by the ethA gene. It interferes with the mycolic acid synthesis by forming an adduct with NAD that inhibits the enoyl-ACP reductase enzyme. EthA is regulated by the transcriptional repressor EthR and its mutation cause resistance to both isoniazid and ethionamide. Moreover, studies with spontaneous isoniazid and ethionamideresistant mutants of *M. tuberculosis* found that they map to mshA, encoding an enzyme essential for mycothiol biosynthesis (Figure 11). Clofazimine (Lamprene) is a riminophenazine compound reported long ago as having anti-TB activity (Figure 8). It is now considered in the group 5 drugs of the WHO for the management of MDR-TB. Clofazimine works by binding to the guanine bases of bacterial DNA, thereby blocking the template function of the DNA and inhibiting bacterial proliferation. Recent studies, however, have signalled the outer membrane as the possible target of clofazimine. Another study found that in M. tuberculosis clofazimine is reduced by NADH dehydrogenase and subsequently after spontaneous re-oxidation liberates bactericidal levels of reactive oxygen species (ROS). Resistance to clofazimine has not yet been fully characterized; however, a recent study has found that in spontaneous mutants of the reference strain H37Rv, mutations in the transcriptional regulator Rv0678 caused an up-regulation of MmpL5, a multi-substrate efflux pump, which not only caused resistance to clofazimine but also to bedaquiline.

Also part of the category 5 drugs of second-line anti-TB drugs, linezolid is an oxazolidinone originally approved for clinical use in the treatment of skin infections and nosocomial pneumonia caused by Gram-positive bacteria. Cycloserine is an oral bacteriostatic secondline anti-tuberculosis drug used in MDR-TB treatment regime. It is an analogue of D-alanine that by blocking the activity of D-alanine: D-alanine ligase inhibits the synthesis of peptidoglycan. It can also inhibit D-alanine racemase (AlrA) needed for the conversion of L-alanine to D-alanine. All these enzymes are involved in bacterial peptidoglycan precursor s synthesis and thus cell wall synthesis was blocked by those antibiotics. Although the actual target of cycloserine in M. tuberculosis is not completely elucidated, in previous studies in *M. smegmatis* it was shown that overexpression of *alrA* led to resistance to cylcoserine in recombinant mutants. More recently, it has also been shown that a point mutation in cycA, which encodes a D-alanine transporter, was partially responsible for resistance to cycloserine in M. bovis BCG. Thioacetazone is an old drug that was used in the treatment of TB due to its favourable in vitro activity against M. tuberculosis and it's very low cost. It has toxicity problems, however, especially in patients co-infected with HIV. It belongs to the group 5 drugs of the WHO and acts by inhibiting mycolic acid synthesis.

Mycobacterium is intrinsically resistant to most commonly used antibiotics. Although high resistance reported worldwide but no MDR conjugative plasmid containing 5-15 mdr genes have been isolated similar to multidrug-resistant Escherichia coli and Pseudomonas aeruginosa. This led to conclusion that specific structure and composition of the mycobacterial cell wall may be important as effective permeability barrier but others have implicated chromosomal drug efflux genes as main culprit for natural resistance. Recent reports have suggested that drug resistant mechanisms must be reinvestigated as day by day a few drugs are active against Mycobacterium increasing STDs in both developed as well as poor countries. Natural macrolides, such as erythromycin as well as semi-synthetic derivatives, such as clarithromycin and azithromycin, are not effective against Mycobacterium tuberculosis although few other species may be sensitive [63]. Other macrolide resistance mechanisms involve antibiotic inactivation, active drug efflux, and mutated ribosome components (ribosomal proteins or 23S rRNA where lincosamide and streptogramin resistance have been reported.

Role of blaTEM-like genes in multi-resistant TB: The 1st mdr gene isolated is blaTEM as in plasmid pBR322 in 1965. When we searched for blaTEM in plasmids of Mycobacterium tuberculosis but no such plasmid was found. It was astonishing but we found few GenBank data as plasmid in other Mycobacterium species like Mycobacterium liflandi (pMUM002; accession no. EU271968) and Mycobacterium avium (pVT2; accession no. AY056023) but no mdr genes found. This indicated that life cycle of Mycobacterium is totally different than most Enterobacteriaceae like E. coli, B subtilis, S. aureus, K. pneumoniae, S. enterica and P. aeruginosa where most plasmids have abundant mdr genes. Next question is conjugation in Mycobacterium may be impossible to acquire the *mdr* genes from water borne bacteria. Neither such organism has colonised in the intestine so that directly inhibited by oral antibiotics and favours conjugation with thousand intestinal MDR bacteria. Then how drug resistant TB was appeared with resistant to most antibiotics available today? The answer is predominance of drug efflux genes in Mycobacterium chromosome similar to the Neiserria gonorrhoeae where penA and ponA genes are highly detected giving resistant to penicillins. Interestingly, we found penP penicillin binding protein in Mycobacterium genomes (protein id: BAX25666, CMT52811, KDM97429, CMR96284, CKY34640 and CMN93417). Seq-2 BLAST search for most Beta-lactamase genes from Enterobacteriaceae (E. coli, S. enterica etc.) did not match blaC beta-lactamase of Mycobacterium tuberculosis [8,64,65]. The accession numbers we tested are given in Table 2.

However, blaC enzyme was cloned, sequenced and over expressed and many beta-lactam drugs were tested for hydrolysis and thus it was authentic BlaC enzyme (Protein ids. ABQ73843, AIU21081 and AIR47299) involved in multi-resistance in Mycobacterium spp. [66-68]. However, seq-2 BLAST protein sequence analysis revealed that BlaC of Mycobacterium tuberculosis was related to class A blaTEM (45%), blaCTX-M-9 (50%) and blaKPC (49%) beta-lactamases and had no similarities to MBL metallo beta-lactamases (IMP, NDM-1, SPM, DHA and GIM) (Table 2). Interestingly, blaTOHO (43%) and blaGES (40%) have also similarities with blaC beta-lactamase of Mycobacterium spp. Mycobacterium abscessus and Mycobacterium tuberculosis class-A beta-lactamases were well documented [69-73]. Thus, beta-lactam drugs may be effective against TB [74,75]. Boronic acid inhibitors also have reported as promising drug to cure beta-lactamase induced XRD-TB [76]. Arabinogalactan, a highly branched polysaccharide, serves to connect peptidoglycan with the outer mycolic acid layer, and a variety of unique glycolsyltransferases are used for its assembly [77]. A new class of anti-mycobacterial agent (1,3-benzothiazin-4-ones) kills Mycobacterium tuberculosis by blocking the enzyme decaprenylphosphoryl- $\beta$ -D-ribose 2'-epimerase which otherwise forms decaprenylphosphoryl arabinose, a key precursor of the cell-wall arabinans, thus provoking cell lysis and bacterial death [78]. Molecular cloning of a beta-lactamase type enzyme in Mycobacterium tuberculosis H37Rv has been described [79]. Interestingly, molecular differences in membrane architecture as discussed above and unusual beta-lactamases in Mycobacterium tuberculosis made beta-lactam antibiotics out of choice for tuberculosis therapy. However, the high cost of carbapenem drugs may be another reason for DOTS drugs inclusion of meropenem and doripenem which described as promising anti-TB drug by many researchers (Table 2) [73,80].

Penicillin-binding protein in *Mycobacterium tuberculosis* genome: Although blaKPC, blaNDM, blaOXA, blaCTX-M were not

BLA protein id	BLA Isomers	Protein id	AA no	Identity	Sequence cover	Expect Value
BlaC,307aa	AMP	AAB59737	286aa	118/263	86%	6.00E-71
33	blaTEM-1	AKA86566	286aa	92/231	80%	1.00E-49
33	blaCTXM-1	CAA63262	291aa	18/261	89%	6.00E-71
33	blaCTXM-2	ATQ37988	291aa	129/294	95%	7.00E-70
33	blaCTXM-9	AAZ30046	291aa	117/262	90%	2.00E-69
33	BlaKPC-1	AAG13410	293aa	119/240	83%	2.00E-72
33	blaNDM-1	AGC54622	270aa	4/6	2%	5.1
33	blaOXA-2	CAC82805	276aa	12/38	13%	0.082
33	blaOXA-23	AET95879	273aa	8/24	8%	4.3
33	blaOXA-48	CDP90224	265aa	8/15	5%	0.063
"	blaOXA-58	AGC92783	280aa	8/15	5%	5.1
33	blaIMP-1	AAB30289	246aa	4/5	2%	1.7
33	blaVIM-1	CAC82722	266aa	15/47	17%	0.1
				15/50	18%	2
33	blaAmp-C	AAD28044	382aa	26/61	24%	0.53
33	blaCMY-1	CAA63264	381aa	11/33	8%	0.006
				20/80	21%	0.65
33	blaSPM	CAD37801	276aa	0/0	0%	-
33	blaACC-1	CAB46491	390aa	8/22	5%	0.76
				10/25/16/53	6%/13%	4.6/8.1
33	blaTOHO-1	BAA07082	291aa	129/298	100%	1.00E-69
33	blaDHA-1	ABN58447	379aa	5/7	2%	5.2
33	blaGES-1	AAF27723	250aa	93/233	93%	2.00E-48
53	blaGIM-1	AEX25999	250aa	5/9	3%	2.4

**Table 2:** Seq-2 BLAST of  $\beta$ -lactamase amino acid sequences compare with blaC protein of *Mycobacterium tuberculosis* (Demonstration of BlaC enzymes of *Mycobacterium tuberculosis* is different than *Enterobacteriaceae* beta-lactamase genes).

reported in MDR *Mycobacterium tuberculosis* except Class A BlaC with homology to blaTOHO and blaTEM, many penicillin binding protein were reported. Figure 12 showed a multi-align data demonstrating the mutations of PBP2 implicated in differential penicillin resistance in *M. tuberculosis*.

Roles of rRNA mutations and methyl-transferases in multiresistant TB: ErmA gene encoding methyl transferase has been located in other members of the *M. tuberculosis* complex, including *M. bovis*, *M. africanum* and *M. microti*. The most common mutations in rRNA gene was found at position 1400 and 1401 of the 16S rRNA (*rrs*) gene, conferring high-level resistance to kanamycin and amikacin, capreomycin and viomycin [81].

**Roles of drug efflux proteins in MDR** *Mycobacterium tuberculosis:* Astonishingly, we also detected emrB drug transporter and MFS drug transporters (protein id. AMP28863) in *Mycobacterium tuberculosis* PR10 genome (accession no. CP010968). The lfrA drug efflux pump was reported first in *Mycobacterium smegmatis* and plays an important role in ciprofloxacin resistance and lfrR is a transcriptional regulator similar to tetR repressor regulating tet operon [82]. Few *M. tuberculosis* drug





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Figure 12: Mutations of specific PBP2 of *Mycobacterium tuberculosis*. Protein sequences were derived from WGS as follows: AUS50827 (accession no. CP024614.1; nt 1955692-1957245 complement); EFD53848 (accession no. DS985189.1; nt.546057-547673; KBX80709 (accession no. JLCA01000005.1; nt. 207622-209256); SGJ61490 (accession no. FPVX01000005.1; nt.29337-30803) and AAK46043 (AE000516.2; nt. 1946550-1948169 complement). (Red means strong homology, green means less homology and black means diverged).

efflux proteins were identified and Rv1258c-type drug efflux conferred resistance to tetracycline and aminoglycosides like streptomycin [83]. Genuinely absence of strA/B and aphA4-type drug phosphorylating genes in plasmid and chromosome is astonishing. But similar unusual assembly of mdr genes was reported in MDR Neisseria gonorrhoeae [5]. Similarly, M. tuberculosis H37Rv epfA protein has similarity to QacA drug efflux super family and reports also suggested Rv2686c-2687c-2688c operon may have multidrug efflux genes [84,85]. ABC transporters like arrA/B also have been characterized in M. tuberculosis [86]. However, acrAB/CD of Escherichia coli and mexAB/CD/EF types of Pseudomonas aeruginosa well documented drug transporters have not reported in Mycobacterium tuberculosis [87,88]. Figure 13 showed the sequence divergence between ermB and qacB drug efflux genes. Figure 14 was disclosed a suspected MFS drug efflux gene in M. tuberculosis where as a suspected drug efflux protein was presented in Figure 15 giving resistant to ciprofloxaci [89].

**Erythromycin resistant gene in** *Mycobacterium:* Erythromycin esterase was located in *Mycobacterium tuberculosis* genome at nt. 2273141-2275186 in the complement orientation (Accession no. CP016794). *ErmMT* Methyl transferase (23S rRNA A<sup>2058</sup>) was present in most members of the Mycobacterium and involved in macrolide resistance and the enzyme was similar in action to monomethyl transferase [81]. Buriankova et al. introduced plasmids with ermMT gene (pOMV16, pOMV20, pOMV30, and pMIP12) and was transformed into the macrolide-sensitive strains, *M. bovis* BCG Pasteur and *M. smegmatis* and the resulting strains showed increase in erythromycin MIC from 8 µg to 1000 µg/ml with pOMV20 ermMT plasmid and to some lesser extent with other plasmids [90].

**Drug acetyl-transferases and phospho-transferses in** *Mycobacterium*: Due to lack of R-plasmids and MDR conjugative plasmids in *Mycobacterium tuberculosis*, catB3, aacC1 and aacA1 types of acetyl transferases have not been linked to multidrug-resistance in Mycobacterium. Most abundant chloramphenicol acetyl transferase has not been documented and my attempt is to make attention in



Figure 13: Sequence similarity between QacB and emrB drug efflux proteins of *Mycobacterium tuberculosis*. (Red means conserved, green means has homology and black signifies no homology).

1	mivqrtaapt	gsvppdrhaa	rpfiprmirt	favpiilgwl	vtiavlnvtv	pqletvgqiq
61	avsmspdaap	smismkhigk	vfeegdsdsa	amivlegqrp	lgdaahafyd	qmigrlqadt
121	thvqslqdfw	gdpltatgaq	ssdgkaayvq	vklagnqges	lanesveavk	tiverlappp
181	gvkvyvtgsa	alvadqqqag	drslqgieav	tftviivmll	lvyrsiitsa	imltmvvlgl
241	latrggvafl	gfhriiglst	fatnllvvla	iaaatdyaif	ligrygearg	lgqdresayy
301	tmfggtahvv	lgsgltiaga	tfclsftrlp	yfqtlgvpla	igmvivvaaa	ltlgpaiiav
361	tsrfgkllep	krmarvrgwr	kvgaaivrwp	gpilvgaval	alvglltlpg	yrtnyndrny
421	lpadlpaneg	yaaaerhfsq	armnpevlmv	esdhdmrnsa	dflvinkiak	aifavegisr
481	vqaitrpdgk	piehtsipfl	ismqgtsqkl	tekynqdlta	rmleqvndig	snidqmermh
541	sltqqmadvt	hemviqmtgm	vvdveelrnh	iadfddffrp	irsyfywekh	cydipvcwsl
601	rsvfdtldgi	dvmtedinnl	lplmqrldtl	mpqltammpe	miqtmksmka	qmlsmhstqe
661	glqdqmaamq	edsaamgeaf	dasrnddsfy	lppevfdnpd	fqrgleqfls	pdghavrfii
721	shegdpmsqa	giariakikt	aakeaikgtp	legsaiylgg	taamfkdlsd	gntydlmiag
781	isalclifii	mlittrsvva	aavivgtvvl	slgasfglsv	liwqhilgie	lhwlvlamav
841	iillavgady	nlllvarlke	eihagintgi	iramggsgsv	vtaaglvfaf	tmmsfavsel
901	tvmaqvgtti	gmgllfdtli	vrsfmtpsia	allgkwfwwp	qvvrqrpvpq	pwpspasart
961	falv					

Figure 14: Amino acid sequence of the MmpL5 siderrophore RND drug transporter involved in bedaquiline and clofazimine resistance in *M. tuberculosis*.

BAX48050	MGARAIFRGFNRPSRVLMINQFGINIGFYMLMPYLADYLAGPLGLAAWAVGLVMGVRNFS M ARAIFRGFNRPSRVLMINOFGINIGFYMLMPYLADYLAGPLGLAAWAVGLVMGVRNFS	60
AUS49914	MXARAI FRGFNRPSRVLMINQFGINI GFYMLMPYLADYLAGPLGLAAWAVGLVMGVRNFS	60
BAX48050	QQGMFFVGGTLADRFGYKPLIIAGCLIRTGGFALLVVAQSLPSVLIAAAATGFAGALFNP OOGMFFVGGTLADRFGYKPLIIAGCLIRTGGFALLVVAOSLPSVL AAAATGFAGALFNP	120
AUS49914	eq:QQMFFVGGTLADRFGYKPLIIAGCLIRTGGFALLVVAQSLPSVIXAAAATGFAGALFNP	120
BAX48050	AVRGYLAAEAGERKIEAFAMFNVFYQSGILLGPLVGLVLLALDFRITVLAAAGVFGLLTV AVRGYLAAEAGERKIEAFAMFNVFYQSGILLGPLVGLVLLALDFRITVLAAAGVFGLLTV	180
AUS49914	AVRGYLAA EAGERKIEAF AMFNVFYQSGILLGPLVGLVLLALDFRITVLAA AGVFGLLTV	180
BAX48050	AQLVALPQHRADSEREKTSILQDWRVVVRNRPFLTLAAAMTGCYALSFQIYLALPMQASI AQLVALPQHRADSEREKTSILQDWRVVVRNRPFLTLAAAMTGCYALSFQIYLALPMQASI	240
AUS49914	AQLVALPQHRADSEREKTSILQDWRVVVRNRPFLTLAAAMTGCYALSFQIYLALPMQASI	240
BAX48050	LMPRNQYLLIAAMFAVSGLVAVGGQLRITRWFAVRWGAERSLVVGATILAASFIPVAVIP LMPRNQYLLIAAMFAVSGLVAVGGQLRITR FAVRWGAERSLVVGATILAASFIPVAVIP	300
AUS49914	LMPRNQYLLIAAMFAVSGLVAVGGQLRITRXFAVRWGAERSLVVGATILAASFIPVAVIP	300
BAX48050	NGQRFGVAVAVMALVLSASLLAVASAALFPFEMRAVVALSGDRLVATHYGFYSTIVGVGV NGQRFGVAVAVMALVLSASLLAVASAALFPFEMRAVVALSGDRLVATHYGFYSTIVGVGV	360
AUS49914	NGQRFGVAVAVMALVLSASLLAVASAALFPFEMRAVVALSGDRLVATHYGFYSTIVGVGV	360
BAX48050	LVGNLAIGSLMSAARRLNTDEIVWGGLILVGIVAVAGLRRLDTFTSGSQNMTGRWAAPR LVGNLAIGSLMSAARRLNTDEIVWGGLILVGIVAVAGLRRLDTFTSGSQNMTGRWAAPR	419
AUS49914	LVGNLAIGSLMSAARRLNTDEIVWGGLILVGIVAVAGLRRLDTFTSGSQNMTGRWAAPR	419
Figure 15: N	Mutation of MFS drug efflux proteins of Mycobacterium tubercu	ılosis

Figure 15: Mutation of MFS drug efflux proteins of *Mycobacterium tuberculosis* involved in fluoroquinolone resistance.

that issue if we have missed such genes in *Mycobacterium*. Evidence was reported that chromosomal acetyl transferases confer kanamycin resistance in *M. tuberculosis* [91,92]. A 2'-N acetyl transferase (aac2'-Ic) was reported in *M. tuberculosis* [93]. Hull et al. reported a plasmid with aminoglycoside transferase but *M. fortuitum* is drug sensitive implying a problem in the study [94]. A phosphotransferase was isolated from *M. fortuitum* involved in streptomycin resistance [95]. Such discrepancy of *mdr* genes between *Escherichia coli* and *Mycobacterium tuberculosis* was reviewed recently [96].

Search for novel chromosomal mdr genes in *Mycobacterium tuberculosis*: Although I have presented a great deal of data supporting the multi-resistance mechanism. The differences in mechanism with the most Enterobacteriaceae are important. So, I have extended such search by looking the entire genome. The complete genome sequences of two *M. tuberculosis* strains, H37Rv and CDC1551 and later hundreds of complete genome sequences have been deposited in the GenBank. I have searched and presented the few suspected *mdr* genes from the whole genome search of *Mycobacterium tuberculosis*. We detected emrB drug transporter and MFS drug transporters (protein id. AMP28863) in *Mycobacterium tuberculosis* PR10 genome (accession no. CP010968). Further, isoniazid drug induced proteins were located like IniA (protein id. BAX25975) and IniC (protein id. BAX25976). Also class A betalactamase like proteins was detected by search (protein id. AMP30076). A total search of *mdr* and drug efflux genes was given below to explain the chromosomal origin of multidrug-resistance but roles of MDRplasmids remain a question (Figure 16) [97].

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All β-lactam antibiotics show structural mimicry to the natural substrates of the bacterial DD-transpeptidases. DD-Transpeptidases, members of the penicillin-binding protein family, catalyze the crosslinking of two peptidoglycan strands which is an essential step in bacterial cell wall biosynthesis. Production of the broad-spectrum Ambler class A β-lactamase BlaC is a major contributor to intrinsic resistance of Mycobacterium tuberculosis to most β-lactams. Combining drugs with a BlaC inhibitor has been recognized for decades as a potential approach for the treatment of tuberculosis. BlaC from Mycobacterium was cloned [69] and over expressed to study the betalactam hydrolysis. BlaC beta-lactamase was highly susceptible to the beta-lactamase inhibitor BRL 42715, with an IC<sub>50</sub> of 0.1 ng/ml and cavulinate is a moderate inhibitor [98]. Mycobacterium BlaC G132N substitution, which restored the canonical SDN motif (SDG→SDN), allowed for efficient hydrolysis of clavulinate, with a more than 104fold increase in  $k_{\rm cat}$  (0.41 s<sup>-1</sup>), without affecting the hydrolysis of other  $\beta$ -lactams like ampicillin (Km=55  $\mu$ M) and meropenem (Km=1  $\mu$ M). Crotonamide derivatives and asperversiamides-ABC were shown as new anti-TB agents [49,50] whereas tetrahydropyridines as QacA drug efflux inhibitors may be useful drug [35].

## Discussion

Mycobacterium tuberculosis infections are increasing due to failure of potent drugs like streptomycin and rifampicin. It appears mutation theory holds promise involving 16S rRNA, rssL and rpo proteins. However, most cases  $\beta$ -lactams were not responding and penA and blaC proteins were abundant in the Mycobacterium chromosome. Conventional cat, strA, strB, aph, aacA1, aacC1, sul1/2/3, arr3, mcr-

001	82487; Acce:	ssion no. CH	2016794, nt	2046897-21	048618.	
001	mwinigviii	svviavrinv	risydduana	langkaregi	asguguvkis	gvigiwmsig
061	VISVMAVINV	trvmadiyit	qriilawrvw	Ituurtdami	agrayyrali	laetlanpaq
121	riqqdvdift	agaggtpnap	sngtastllf	gavqsiisvi	sitailwnis	gtlnifgvsi
181	pramfwtvlv	yvfvatvisf	iigrpliwls	frneklnaaf	ryalvrlrda	aeavgfyrge
241	rvegtqlqrr	ftpvidnyrr	yvrrsiafng	wnlsvsqtiv	plpwviqapr	lfagqidfgd
301	vgqtatsfgn	ihdslsffrn	nydafasfra	aiirlhglvd	anekgralpa	vltrpsddes
361	velndievrt	pagdrlidpl	dvrldrggsl	vitgrsgagk	ttllrslael	wpyasgtlhr
421	pggenetmfl	sqlpyvplgt	lrdvvcypns	aaaipdatlr	dtltkvalap	lcdrldeerd
481	wakvlspgeq	qrvafarill	tkpkavflde	stsaldtgle	falyqllrse	lpdcivisvs
541	hrpalerlhe	nqlellgggq	wrlapveaap	aev.		-
MFS	Transporte	r (AmiC); pi	rotein id. A	NZ83566 (4	70aa); Acces	ssion no.
CPO	16794, nt. 3	3186584-3188	3005.			
001	msrvhafvdd	algdldaval	adairsgrvg	radvveaaia	raeavnpaln	alayaafdva
061	rdaaamgtgq	eaffsgvptf	ikdnvdvagq	psmhgtdawe	pyaavadsei	trvvlgtglv
121	slgktqlsef	qfsavaehpr	lqpvrnpwnt	dytagasssg	sgalvaagvv	piahandqqq
181	siripaacnq	lvglkpsrgr	lplepeyrrl	pvgivangvl	trtvrdtaaf	yreaerlwrn
241	hqlppvqdvt	spykgrlria	vvtrsvlrea	spevrqltlk	laglleelgh	rvehvdhppa
301	pasfyddfyl	vwgflalagy	rsgrrtfart	fdptrldelt	lglarhtgrn	lhrlplaimr
	lrmlrrrsvr	ffgtvdvllt	ptvaeatpgv	gylaptdygt	vldrlsswvv	ftpygnytgy
361			andt grant 1	lelavelees	rnwarihann	iae
361 421	paislplags	addmpydmmi	Saucurearr	TETUVETEEd	TOWGTTHRDH	THE.

no. CP016794).

1, blaNDM-1, blaOXA-1, CTX-M1/2/9 enzymes have not detected in plasmids of *Mycobacterium* (Figure 17). In truth MDR conjugative plasmids have not reported demonstrating host specific *Mycobacterium* is less prone to share genetic material by conjugation. Amikacin and linezolid are promising drugs whereas levofloxacin, meropenem are in clinical trials but optimistically multidrug-resistant TB cases are in the rise worldwide. I speculate that MDR plasmids will be in *Mycobacterium* but due to low copy number and heterogeneities among them, careful asses must be needed. Critic will rule out my statement but I hope further calamity will force us to do work carefully. DOTS antibiotics are: group 1 (Isoniazid, rifampicin, ethambutol, pyrazinamide), group 2 (streptomycin, amikacin, kanamycin,capreomycin); group 3 (ciprofloxacin, ofloxacin, levofloxacin, moxifloxacin, gatifloxacin and group 4 (ethonamide, prothionamide, cycloserine, terizadone, paraamino salycilic acid, thiacetazone) [61].

TB treatment is lengthy, isoniazid+ rifampicin+ pyrazinamide + ethambutol for two months followed by isoniazid + rifampicin for next four months (total six months). So many drugs we ingest, your total gut bacteria will be finished and thus vitamin supplement is must during therapy to survive. Certain cases weekly regime is followed due to toxicity of drugs. As we discussed, all these drugs may not work and WHO third line drugs like clarithromycin, linezolid, thioridazine and bedaquiline. The rate of spontaneous mutation was reported where 1 mutation for every 107 cell division for streptomycin and isoniazid and  $10^{10}$  for rifampicin. Patients with extensive pulmonary TB have ~ $10^{12}$ bacteria and thus changes for rifampicin + isoniazid resistant bacteria will be 1 in 108 and all four drug resistant (rifampicin + isoniazid + streptomycin + ethambutol) will be 1 in 10<sup>33</sup>. Even such calculation may not explain the correct outcome regarding MDR-TB but I argue DOTS therapy is simply toxic and generating more complex microbes and also limits the life of patients. If we find a drug that has specific target, then simply one drug will kill the bacteria completely in one day if the drug reach the target and if the bacteria is replicating. Central nervous system-TB and bone-TB may need cautious approach where steroids (prednisolone, dexamethasone) may be useful and thalidomide may be beneficial to TB meningitis. On October 2006, the XRD-TB means resistant to fluoroquinolones and any of one drug from kanamycin, capreomycin and amikacin [99]. Kanamycin resistant is overwhelming in MDR Enterobacteriace isolates due to diversified phosphotransferases and overwhelming mutation in the gyrA and gyrB



subunits of DNA gyrase as well as topoisomerase IV. That way 95% TB clinical isolates should be multidrug-resistant but that was not the case. Because aminiglycoside resistance in *Mycobacterium* has shifted to mutation of 16S rRNA and ribosomal proteins. No plasmid and integron were reported for *M. tuberculosis*. Then why we have not use cefotaxime and meropenem in TB therapy? Likely similar to gonococcus bacteria beta-lactams drugs did not showed promise in clinical therapy, possibly due to penA penicillin binding protein in *Mycobacterium* chromosome and may be activated by drugs similar to blaAmp-C beta-lactamase or tetA/C genes by tetracycline (Figure 17) [100].

Ethambutol inhibits TB bacilli by inhibiting arabinosyl transferase to form mycolic acids attach to the 5'-hydroxyl groups of D-arabinose residues of arabinogalactan making mycolylarabinogalactan-peptidoglycan complex in the cell wall [54]. Mechanism of MDR-drug, pyrazinamide is not clear but pyrazinoic acid was thought to inhibit the enzyme fatty acid synthase I (FAS), which is required by the bacterium to synthesize fatty acid and also binds to the ribosomal protein S1 (rpsA) and inhibit trans-translation [53]. Mutations in the pncA gene may be responsible for the majority of pyrazinamide resistance in M. tuberculosis strains. Peptide antibiotics like capreomycin was discovered from Streptomyces [96] capreolus in 1960 and now widely used in case of MDR-TB as also veomycin. Several mycobacterial efflux pumps associated with fluoroquinolone resistance have been described, including pumps of the MFS family (lfrA and Rv1258c) and ABC transporters (DrrAB, PstB and Rv2686c-2687c-2688c). A new class of spectinomycin analog, spectinamide evaded Rv1258c over-expression through structural modification and restored MICs to susceptible levels by binding to 16S bacterial ribosomal subunit. Various mutations like P1098L, G198A and C213A within Rv0194, Rv1634, and Rv2688c EPs respectively has been reported where fluoroquinilone resistant lacks gyrA, gyrB and DNA topoisomerase IV mutations [88]. As discussed above few MFS drug efflux proteins located in whole genome sequence of Mycobacterium tuberculosis (accession nos. MP018036 and CP024614). Amino acid sequence of such MFS drug efflux protein is unrelated to acrA or mexA MFS proteins of Escherichia coli and Pseudomonas aeruginosa respectively and may be used to discover as Mycobacterium specific drug efflux inhibitors [51]. Chinese herb medicine has potential as anti-tuberculosis drug [101]. Over all, too many drugs have to be given to clear MDR-TB. Any chemical in excess is toxic to >30,000 enzymes in our cells and sometime carcinogenic. Thus, during therapy a wellknown nutritionist must help a patient otherwise treatment will bring death even your TB bacilli no longer active in your body. We demand though, a careful and systematic study of Mycobacterium tuberculosis MDR plasmids. Mycobacterium liflandi and Mycobacterium avium plasmids (pMUM002 and pVT2) with no mdr gene may be an indicator justifying our demand. Surely phage therapy, enzybiotics and nanodrug carriers are emerging technologies to curve MDR pathogenesis but I believe on herbal drugs as described in Sastruta Samhita and Atharva Veda according to ancient Hindu Civilization [64,101].

#### Conclusion

The mechanism of AMR in *Mycobacterium tuberculosis* is complex as the resistance is generated against TB-specific drugs. In truth, all classical antibiotics are used against MDR Enterobacteriaceae, have been attempted against TB with moderate success. Thus, generation of *catB3*, *strAB*, *sul1/2*, *blaKPC*, *blaOXA-48*, *blaOXA-51*, *dhfr*, *tetA/C*, *qnrA1*, *acrAB*, *mexCD* are expected but no such mdr genes nor 50-500kb MDR conjugative plasmids are reported till date in *M. tuberculosis*. Genome wide search indicated blaC, PBPs, MFS drug efflux proteins

in chromosome. Likely, BlaC, PBPs, gyrAB and Dcm/Dam MTase mutations might be implicated in penicillins, fluoroquinolones and macrolides resistance. The slow cell division may be the main reason but study indicated that acquisition of too many transposable elements would be created multiple plasmids in Enterobacteriaceae and in M. avium subsp. hominis suis TH135 (135kb plasmid: pMAH135), in M. marinum (114kb plasmid, pRAW) and in M. kansasii ATCC 12478 (145kb plasmid, pMK12478). Likely, high copy number plasmids in M. tuberculosis were absent causing a delay of discovery of MDR plasmids. Otherwise, may be such plasmids are not stable due to very slow replication where replication of plasmids depend on chromosome replication. Surely M. tuberculosis conjugation has been proved today using conjugative plasmids from other species but a real discovery of MDR conjugative plasmid in Mycobacterium tuberculosis will be challenging! Similarly, mutation in the target TB-mdr genes indicated transposition events are random creating a new trait giving drug void. Thus MDR-TB will be increasing and new drug development is an argent need. Mptb gene cloning in expression vector (antisense RNA/ Ribozyme) or alkaloids like seconeolitsine against M. tuberculosis topoisomerase may give new direction for TB control.

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