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Molecular Aspects

Lack of association of novel mutation Asp397Gly in *aftB* gene with ethambutol resistance in clinical isolates of *Mycobacterium tuberculosis*

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ABSTRACT

To discover additional genotypic indicators for ethambutol (EMB) resistant *M. tuberculosis*, we studied polymorphisms in arabinofuranosyl transferase encoding genes *aftA* (*Rv3792*), *aftB* (*Rv3805*) and *aftC* (*Rv2673*) in 38 EMB resistant and 34 EMB susceptible isolates from India and a repository established by the World Health Organization (WHO) Special Programme for Research and Training in Tropical Disease (TDR) by DNA sequencing. The results were correlated with the minimum inhibitory concentration (MIC) of EMB and mutations in *embB* (Rv3795).

The most common non-synonymous polymorphism identified in *aftB* was Asp397Gly in 12/38 (31.6%) EMB resistant and 3/34 (8.8%) EMB susceptible isolates. Interestingly, 10/12 (83.3%) EMB resistant isolates with *aftB* Asp397Gly mutation also carried *embB306*, *embB402* or *embB497* mutations. Association of Asp397Gly polymorphism with EMB resistance was statistically significant (p 0.0216). However, overexpression of the mutant *aftB* in *M. tuberculosis* H37Rv did not exhibit any change in the MIC. Whole genome sequencing of a panel of Indian isolates and SNP cluster grouping (SCG) of TDR strains revealed an association between *aftB* mutation Asp397Gly and Beijing genotype or SCG2, a cluster group representing the Beijing genotype.

To conclude, though *aftB*Asp397Gly mutation is not associated with EMB resistance, this mutation may be a phylogenetic marker for the Beijing clade.

1. Introduction

Ethambutol (EMB), an anti-tuberculosis drug, targets the cell wall, specifically the arabinogalactan and lipoarabinomannan synthesis pathway of *M. tuberculosis* [1,2]. This pathway is instrumental in the formation of a mycobacterial arabinogalactan core which is further tethered to mycolic acid to give the mycobacterium a rigid and characteristic cell wall [3]. The galactan core of the arabinogalactan is synthesized with the help of *ubiA* (*Rv3806*), *dprE1* (*Rv3790*) and *dprE2* (*Rv3791*) genes which sequentially convert phosphoribose-diphosphate (PRPP) to Decaprenylphosphoryl-β-D-arabinose (DPA). The *embCAB, aftA, aftB* and *aftC* genes encode arabinofuranosyl transferases that polymerize arabinofuranosyl (Ara*f*) residues from DPA into the arabinan component of arabinogalactan and lipoarabinomannan [4]. Mutations in the *embCAB* are responsible for resistance to EMB in approximately 60% cases. Though various studies have shown predominance of *embB* mutations, especially the canonical mutations in codons 306, 406 and 497 [5–8] in EMB resistant clinical isolates, the relevance of other genes of the arabinogalactan-lipoarabinomannan pathway are lesser explored. Recent studies have shown that mutations in the upstream region of *embA* (*Rv3794*) such as −8, −11, −12, −16 and −43 may be related to EMB resistance, both exclusively or when present along with canonical *embB* mutations [9,10]. Mutations in *ubiA* have been shown to be associated with EMB resistance in clinical isolates [11,12] while resistance causing mutations were not found in isolates from India [13].

The gene *aftB* is a putative arabinofuranosyl transferase and is responsible for the transfer of Ara*f* residues from DPA to the arabinan

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domain to form terminal β (1 → 2)-linked Araf residues [14]. Its relevance in *M. tuberculosis* has been elucidated through deletion studies in the closely related species *Corynebacterium glutamicum* revealing a decreased abundance of cell wall-bound mycolic acids in the deletion mutants of *aftB*, consistent with a partial loss of mycolylation sites [14].

In the present study, with an aim to find additional regions harbouring EMB resistance causing mutations, besides *embCAB* operon, we explored the polymorphisms in the arabinofuranosyl transferases *aftA, aftB* and *aftC* involved in arabinogalactan biosynthesis pathway, for their association with EMB resistance and *embB* mutations in clinical isolates of *M. tuberculosis.* We also constructed strains of *M. tuberculosis* H37Rv carrying mutant allele Asp397Gly of *aftB*, a novel mutation found in EMB resistant isolates of *M. tuberculosis*, and compared the minimum inhibitory concentration (MIC) for EMB of the recombinant strain and wild-type H37Rv strain, in order to study the association of this mutation with EMB resistance.

2. Material and methods

2.1. Mycobacterium tuberculosis isolates

The study included all EMB resistant isolates (n = 38) and randomly selected 34 EMB susceptible isolates of *M. tuberculosis* obtained from two independent sets of samples (Supplementary Tables 1 and 2). The first set of isolates was from India, comprising of 28 EMB resistant and 29 EMB susceptible isolates of *M. tuberculosis* selected from a previously published study [13], collected from new smear positive patients of pulmonary tuberculosis over a 5-year period from January 2011 to December 2015. Informed consent and detailed history of contact were taken from each patient prior to the collection of samples as described previously [13], following clearance from the Institutional Ethical Committee. All isolates had been previously characterized as *M. tuberculosis* [13].

A second set of samples, consisting of 10 EMB resistant and 5 EMB susceptible *M. tuberculosis* strains belonging to different geographic regions was selected from a well characterized repository established by the World Health Organization Special Programme for Research and Training in Tropical Disease [4,11,15].

2.2. Minimum inhibitory concentration (MIC)

EMB was obtained from Sigma Aldrich (St. Louis, MO, USA) and stock solutions were prepared in deionized water. The MIC of the Indian isolates was tested by broth microdilution Microplate Alamar Blue Assay (MABA) [13]. The strains were considered susceptible if their MIC was ≤ 2 μg/ml, low-level resistant (LLR) if their MIC was > 2μg/ml- < 5 μg/ml and high-level resistant (HLR) if the MIC was ≥ 5 μg/ml as previously described [13,16].

MICs of the TDR strains were determined by 7H10 agar proportion and by BACTEC 460 TB methods (Becton Dickinson and Company, Sparks, MD) following the manufacturer's instructions with minor modifications [5]. The same inoculum that was used for the BACTEC460 TB vial was also inoculated onto plates of 7H10 medium containing serial concentrations of antibiotics [17].

Since each value within a triplicate test was almost always identical to the other values within the same triplicate set, a single MIC value is mentioned without standard deviation.

2.3. Determination of polymorphisms

2.3.1. DNA extraction and PCR amplification

Mycobacterial DNA was extracted from culture isolates as described previously [18]. Independent primers were synthesized for amplification of the entire *aftA* and *aftB* genes of the Indian and TDR strains; and *aftC* gene of the Indian isolates (Table 1). The primers of each gene were designed using Suite for Computational identification of Promoter Elements/SCOPE version 2.1.0, such that the predicted promoter region of *aftA* (−51 to −45 and −30 to −24 on the + strand); *aftB* (−15 to −10 and −44 to −39 on the + strand) and *aftC* (−100 to −110 on + strand) were included in their respective amplicons.

The amplified products were gel purified using Gel Extraction Kit (MDI membrane technologies) as per the manufacturer's instructions.

2.3.2. Sanger sequencing

Sanger sequencing of the amplified products was performed using Applied Biosystems 3100 or 3130 Genetic Analyzer. Briefly, the gel-purified amplicons were used in a sequencing PCR using BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). The primers used for the sequencing reaction are given in Table 1. Purification was performed using HighPrep DTR magnetic beads (MAGBIO) prior to sequence analysis by the genetic analyzer, as per the manufacturer's instructions. Sequence alignment and determination of polymorphism was done using BioEdit version 7.2 and NCBI BLAST using the genome of *M. tuberculosis* H37Rv as a reference to determine SNPs [19,20].

2.4. Cloning of mutant and wild type aftB gene and overexpression in M. tuberculosis H37Rv

The overexpression strains, i.e. H37Rv expressing the Asp397Gly mutation of *aftB* (↑*aftB*-Mut) and H37Rv overexpressing wild type (WT) *aftB* (↑*aftB*-WT) were constructed using the mycobacterial shuttle vector pSTK [21]. Briefly, *aftB* gene (1.88 kb) containing the Asp397Gly mutation (*aftB*-MT) was amplified from the clinical isolate ASTR13/13, while the WT gene (*aftB*-WT) was amplified from the laboratory strain H37Rv, using the primers, pST-K *aftB*-F and pST-K *aftB*-R with restriction sites for *Nde*I and *Hin*dIII respectively (Table 1). The amplicons and the shuttle vector pST-K were digested with *Nde*I and *Hin*dIII (New England Biolabs, Beverely, MA, USA) in separate reactions. The digested amplicon and vector were ligated by T4 DNA ligase (New England Biolabs, Beverely, MA, USA) to create recombinant plasmids, pSTK:aftB-Mut and pSTK:*aftB*-WT which were transformed in *E. coli* DH5α cells and the transformants selected on Luria-Bertani (LB) agar containing 25 μg/ml kanamycin. The clones were confirmed by PCR amplification and plasmid digestion. Presence or absence of the Asp397Gly mutation was verified by sequencing. The recombinant plasmids were separately electroporated into competent cells of *M. tuberculosis* H37Rv, plated onto 7H11 (Becton Dickinson and Company, USA) plates supplemented with 25 μg/ml kanamycin &10% OADC (Becton Dickinson and Company, USA) and incubated for 3–4 weeks at 37 °C until the appearance of bacterial colonies. The recombinant constructs were confirmed for protein expression by performing western blot analysis using primary anti-flag antibody (anti-flag mice IgG, Abcam, Cambridge, MA) and secondary antibody (peroxidase conjugated anti mouse IgG, Abcam, Cambridge, MA) according to the manufacturer's instructions. Development was done using 0.5 mg/ml 3,3′ diaminobenzidine tetrahydrochloride (DAB) (Sigma- Aldrich, USA) dissolved in phosphate buffer saline (PBS) and 30% Hydrogen Peroxide (H_2O_2). *M. tuberculosis* H37Rv electroporated with empty vector pSTK was used as control. The overexpression of mutated *aftB* in the recombinant strain (↑*aftB*-Mut) was also assessed using real-time PCR (Light Cycler 480II, Roche). *M. tuberculosis* H37Rv was used as the control and *sigA* was used as an internal control for the analysis.

2.5. Determination of EMB MIC in the recombinant strains

The MIC of EMB for both the overexpression strains, ↑*aftB*-Mut and ↑*aftB*-WT as well as *M. tuberculosis* H37Rv was assessed using MABA assay. The assay was performed using 96 wells U bottom plates in triplicates for each concentration (0.5 μg/ml to 64 μg/ml) and was repeated a minimum of three times.

Table 1
Primers used in the study for cloning and sequencing.

Gene	Experimental set	Gene Length	Amplicon length	Primer Sequence
aftA (Rv3792)	India	1932bp	2200bp	**FP 5′AGGTGCGTACCCGGAT3′** FP 5′CCACCGTTTTACCCACC3′ FP 5′GCTGTGGCTGGTGATGC3′ **RP 5′GCACGGAGGTAGATGGTAG3′**
aftA (Rv3792) [4]	TDR	1932bp	2163bp	F1-3792Seq **5′CGCACCTCAAGGAAGCTCCA3′** R11-3792Seq **5′CGCACGGAGGTAGATGGT3′** F12-3792Seq 5′GCGTTCACGGTGGTGCTGAT3′ F-BCN3P-3792 5′CGATCGGACCGTCGTGTTGA3′ R12-3792Seq 5′AGCCAGGTGGTGGATCCGAT3′ R-BCNWT-3792 5′CCAGCAAGGCGTATTCGAAG3′
aftB (*Rv3805*)	India	1884bp	2100bp	**FP 5′AGCCTCAAGCTCAACGT3′** FP 5′ATGATGGTCTGCTGGTCA3′ FP 5′GTCATTCCTATTTTGCTGC3′ **RP 5′GATCTTGCTGTCAGTATT3′**
aftB (*Rv3805*) (TDR strains)	TDR	1884bp	2129bp	**Fragment 1** **F-3805seq 5′CATGTGTCGACCGCTAGTGC3′** **R1-3805seq 5′CCGCTGGGATTGCTGTTGA3′** **Fragment 2** **F2-3805seq 5′CCGACGATGAAAGCCACCA3′** **R1-3806CL 5′GAAGATATCGCGCTGCGTG3′**
aftC (*Rv2673*)	India	1302bp	1470bp	**FP 5′GTGATGTGGCAAGGTATTG3′** **RP 5′ATCACCGCTAGTCCCGA3′**
*aftB** (*Rv3805*) (over-expression studies)	India	1884bp	1897bp	**pSTK-*aftB*F 5′GTCATATGGTCCGGGTCAGCTTGTG3′** **pSTK-*aftB*R 5′ATAAGCTTTAACTCCCGCGGTGGC3′**

The primer sequences in bold were used for PCR amplification. Underlined sequences were the primers used in sequencing reactions.

2.6. Determination of the genotype of TDR strains by SNP cluster group (SCG) analysis

SCG analysis used nine SNP markers [22] derived from a combination of previously described SNPs based on whole genome comparisons of *M. tuberculosis* H37Rv, CDC1551 and 210; and *M. bovis AF2122/97* [19,23–26]. Each *M. tuberculosis* TDR isolate in the present study was assigned to one of seven phylogenetically distinct SCG groups (SCG 1–7) and five subgroups (SCG 3a, 3b, 3c, 6a, 6b) using the SNP markers as described previously [22,26]. The allele of each SNP marker was identified using hairpin primer PCR assays [27].

2.7. Whole genome sequencing of a panel of isolates

The genomic polymorphisms and the genotype were confirmed by whole genome sequencing performed on 27 EMB resistant and 22 EMB susceptible Indian isolates. DNA was prepared for sequencing with the Illumina Nextera XT DNA Library Prep kit and paired-end sequenced on an Illumina NextSeq 500 platform according to the manufacturer's instructions. Each strain was sequenced to obtain > 99% coverage relative to the *M. tuberculosis* H37Rv reference genome, with an average depth of at least 50× as previously described [28]. The sequences reported in this paper have been deposited in the Sequence Read Archive of the National Centre for Biotechnology Information under study accession number SRP154957.

3. Results

3.1. Determination of minimum inhibitory concentration of M. tuberculosis clinical isolates to EMB

The MICs of the clinical isolates included in the study have been described in detail previously [4,11,13]. Briefly, all the strains were characterized into one of three EMB MIC categories: Susceptible (MIC 2 μg/ml), low level resistant (MIC 4 μg/ml) and high level resistant (MIC ≥ 8 μg/ml). MICs were determined for 24 EMB susceptible and 24 EMB resistant Indian isolates (Tables 2 and 3). Of the EMB resistant isolates, 4/24 (16.7%) were low level resistant, while 20/24 (83.3%) were high level resistant to EMB [12]. In the isolates belonging to the TDR group, 5 isolates were susceptible, 2 isolates were low level resistant and 8 isolates were high level resistant to EMB (Tables 2 and 3) [4,11].

3.2. Determination of polymorphisms in aftA, aftB and aftC in M. tuberculosis isolates

3.2.1. Polymorphisms in aftA

Polymorphisms at *aftA* gene were analysed by Sanger sequencing and/or whole genome sequencing in all the 38 EMB resistant and 26 EMB susceptible Indian and TDR isolates (Tables 2 and 3). The non-synonymous mutations Val70Ile and Ala456Val were observed in 1/26 (3.8%) and 3/26 (11.5%) EMB susceptible isolates (Table 2) respectively and none of the EMB resistant isolates. No mutation was observed in the predicted promoter or the upstream region of the gene.

3.2.2. Polymorphisms in aftB

Polymorphisms in *aftB* were studied in all the 38 EMB resistant isolates and 34 EMB susceptible isolates (Tables 2 and 3). The most notable mutation in *aftB* was Asp397Gly, which was present in 12/38 (31.6%) EMB resistant isolates and 3/34 (8.8%) EMB susceptible isolates (Tables 2 and 3) (p 0.0216; Fisher's exact test). This mutation was also accompanied by Ala83Thr change in 2/38 (5.3%) EMB resistant isolates. Of the EMB resistant isolates with *aftB* Asp397Gly mutation, MIC results were available for 11 isolates. Of these, 3/11 (27.3%) isolates with Asp397Gly mutation had low level resistance to EMB, whereas 8/11 (72.7%) were high level EMB resistant (p 0.36; Fisher's exact test). SNP analysis of the upstream region did not find any mutation in the predicted promoter of *aftB*.

3.2.3. Polymorphisms in aftC

Polymorphism analysis of *aftC* in 27 EMB resistant and 23 EMB susceptible Indian isolates showed the non-synonymous mutation Arg25His only in 1/27 (3.7%) EMB resistant isolate (Table 3). The predicted promoter region also did not contain any polymorphisms. SNP analysis of *aftC* was not available for the TDR isolates.

Table 2
Polymorphisms in the arabinofuranosyl transferase encoding genes of arabinogalactan and lipoarabinomannan synthesis pathway in EMB susceptible *M. tuberculosis* isolates.

S. No.	Strain ID	Geographical origin	Lineage[a]	MIC (μg/ml)[b]	*embB*[b]	*aftA*	*aftB*	*aftC*
1	ASTS1/11	India	EAI	–	Glu378Ala	WT	WT	Ala131Ala
2	ASTS2/11	India	–	–	WT	–	WT	–
3	ASTS3/11	India	–	–	WT	–	WT	–
4	ASTS4/11	India	–	–	WT	–	WT	–
5	ASTS5/11	India	–	–	WT	–	WT	–
6	ASTS6/11	India	Delhi/CAS	1	WT	WT	WT	WT
7	ASTS7/11	India	EAI	1	Glu378Ala	WT	WT	Ala131Ala
8	ASTS8/11	India	Delhi/CAS	1	WT	WT	Ala485Glu	WT
9	ASTS9/11	India	Delhi/CAS	1	WT	WT	Pro423Ser	WT
10	ASTS10/11	India	Delhi/CAS	1	WT	WT	WT	WT
11	ASTS11/11	India	EAI	1	Glu378Ala	Gln63Gln	WT	WT
12	ASTS12/11	India	–	1	Glu378Ala Glu504Asp	–	WT	–
13	ASTS13/11	India	–	< 0.5	WT	–	WT	–
14	ASTS14/11	India	Delhi/CAS	1	WT	WT	WT	WT
15	ASTS15/11	India	Haarlem	2	WT	Ala456Val	WT	WT
16	ASTS16/13	India	Delhi/CAS	2	WT	WT	WT	WT
17	ASTS17/13	India	Delhi/CAS	2	WT	WT	WT	WT
18	ASTS18/13	India	EAI	1	Glu378Ala	WT	WT	Ala131Ala
19	ASTS19/13	India	Delhi/CAS	1	WT	WT	WT	WT
20	ASTS20/13	India	Delhi/CAS	1	WT	WT	WT	WT
21	ASTS21/13	India	EAI	1	Glu378Ala	WT	WT	Ala131Ala
22	ASTS22/13	India	–	1	WT	WT	WT	WT
23	ASTS23/13	India	LAM	1	WT	WT	WT	WT
24	ASTS24/13	India	Delhi/CAS	1	WT	WT	Ser238Cys	WT
25	ASTS25/13	India	Beijing	< 0.5	WT	WT	Asp397Gly	Arg223Arg
26	ASTS26/13	India	EAI	1	Asp311Phe Glu378Ala	WT	WT	Ala131Ala
27	ASTS27/13	India	Haarlem	2	WT	Ala456Val	WT	WT
28	ASTS28/13	India	Haarlem	0.5	WT	Ala456Val	WT	WT
29	ASTS29/14	India	Delhi/CAS	2	WT	WT	WT	WT
30	TDR81	Korea	SCG2	2	WT	Val70Ile	Asp397Gly	–
31	TDR83	Korea	SCG2	2	WT	–	Asp397Gly	–
32	TDR29	Azerbaijan	SCG5	2	WT	His344His	WT	–
33	TDR33	Belgium	SCG6a	2	Ala1073Ala	–	WT	–
34	TDR182	Cameroon	SCG5	2	WT	WT	WT	–

[a] Lineage of the *M. tuberculosis* isolates from India was determined by whole genome sequencing and of the TDR by SNP cluster grouping.
[b] Details given in References [4,11,13].

3.3. Correlation with polymorphisms in embB

The mutations in the *embB* gene in all the EMB resistant and EMB susceptible Indian and TDR isolates have been reported previously [11,13]. Briefly, of all the isolates tested, 27/38 (71%) EMB resistant isolates had a Met306Val/Ile mutation, 3/38 (7.9%) carried a Gln497Arg mutation and 1/38 (2.6%) carried a Leu402Val polymorphism [11,13]. Of the 12 EMB resistant isolates with the *aftB* mutation Asp397Gly, 8/12 (66.7%) also had the canonical mutation Met306Val/Ile at *embB*; 1/12 (8.3%) each had canonical mutations Leu402Val or Gln497Arg at *embB*, while 2/12 (16.7%) LLR isolates only carried the mutation Asp397Gly (Table 3).

3.4. Effect of overexpression of aftB-Mut and aftB-WT in M. tuberculosis H37Rv

We overexpressed the *aftB* mutant (Asp397Gly) and *aftB* wild type (Asp397Asp) alleles and obtained the *M. tuberculosis* H37Rv (pST-K:*aftB*-Mut) strain ↑*aftB*-Mut containing the Asp397Gly allele and *M. tuberculosis* H37Rv (pST-K:*aftB*-WT) strain ↑*aftB*-WT containing the Asp397Asp allele. DNA sequencing of plasmid DNA extracted from both the strains confirmed that the WT and mutant *aftB* genes were successfully inserted into the vector, the expected alleles were present and no additional mutations had been introduced in the *aftB* gene during the cloning process. Western blot analysis of the cell lysate from ↑*aftB*-Mut and ↑*aftB*-WT also confirmed overexpression of the WT and mutant *aftB* gene (Fig. 1). The expression of *aftB*-Mut in ↑*aftB*-Mut strain was found to be up-regulated with respect to the WT *aftB* in *M. tuberculosis* H37Rv when compared using real-time PCR, thus confirming the overexpression of the mutant allele. MICs for EMB in *M. tuberculosis* H37Rv and both the recombinant strains ↑*aftB*-Mut and ↑*aftB*-WT were tested by the MABA assay in triplicates and determined to be 1 μg/ml. Our results, thus, failed to show any effect of the *aftB* mutation Asp397Gly on the EMB MIC of a susceptible strain with wild type *embB* gene.

3.5. Genotyping for lineage classification

3.5.1. SNP cluster groups

Nine SNP markers were analysed in the 15 TDR clinical isolates of *M. tuberculosis* to assign them to one of the known SNP cluster groups (SCG) (Tables 2 and 3). Of these, 7/15 (46.7%) isolates belonged to SCG 2 of which 5/7 (71.4%) were EMB resistant. SCG 5 was observed in 6/15 (40%) isolates and 4/6 (66.7%) of these were EMB resistant. Of all the isolates, 1/15 (6.7%) belonged to SCG 6a and was EMB susceptible. A single EMB resistant isolate was found to be SCG 6b. Analysis of the *aftB* mutation Asp397Gly revealed a strong association with SCG 2. The results showed that 7/7 (100%) of the TDR isolates with the Asp397Gly mutation belonged to SCG 2, a cluster group strongly associated with Beijing genotype.

3.5.2. Whole genome sequencing

Whole genome based phylogenetic analysis of the Indian isolates was performed on 27 EMB resistant and 22 EMB susceptible isolates. Of the isolates tested, maximum number (23/49; 46.9%) of isolates belonged to the Delhi/CAS lineage followed by 9/49 (18.4%) belonging to

Table 3
Polymorphisms in the arabinofuranosyl transferase encoding genes of arabinogalactan and lipoarabinomannan synthesis pathway in EMB resistant *M. tuberculosis* isolates.

S. No.	Isolate No.	Geographical Origin	Lineage[a]	MIC (μg/ml)[b]	*embB*[b]	*aftA*	*aftB*	*aftC*
1	ASTR1/11	India	Beijing	64	Met306Val	WT	Asp397Gly	Asp75Asn/Asp
2	ASTR2/11	India	Delhi/CAS	16	Met306Ile	WT	WT	WT
3	ASTR3/11	India	Euro-American superlineage	4	Glu378Ala	WT	WT	Ala131AlA
4	ASTR4/11	India	Delhi/CAS	–	Met306Val	WT	WT	WT
5	ASTR5/11	India	Delhi/CAS	8	Met306Val	WT	WT	WT
6	ASTR6/11	India	Undetermined	> 64	Met 306Ile	WT	WT	WT
7	ASTR7/11	India	–	32	Ala505Ala	WT	WT	–
8	ASTR8/11	India	EAI	4	Glu378Ala	Gln63Gln	WT	WT
9	ASTR9/11	India	Delhi/CAS	16	Met306Val	WT	WT	Arg25His
10	ASTR10/11	India	Delhi/CAS	8	Gln497Arg	WT	WT	WT
11	ASTR11/11	India	EAI	4	Glu378Ala	Arg489Arg	Ser469Leu	Ala131Ala
12	ASTR12/11	India	Euro-American superlineage	8	Asp345Asp Gln497Arg	His344His	WT	WT
13	ASTR13/13	India	Beijing	32	Met306Val	WT	Asp397Gly	WT
14	ASTR14/13	India	Delhi/CAS	8	Asp328His Asp354Ala	WT	WT	WT
15	ASTR15/13	India	Beijing	16	Leu402Val	WT	Asp397Gly	Arg223Arg
16	ASTR16/13	India	Beijing	16	Met306Val	WT	Asp397Gly Ala83Thr	Arg223Arg
17	ASTR17/13	India	Euro-American superlineage	8	Met306Val	Ala20Ala	WT	WT
18	ASTR18/13	India	EAI	16	Met306Val Glu378Ala	Gln63Gln	Val352Val	Asp75Asn/Asp
19	ASTR19/13	India	Delhi/CAS	8	Met306Val	WT	Val352Val	WT
20	ASTR20/13	India	Delhi/CAS	8	Met306Val	WT	Val352Val	WT
21	ASTR21/14	India	Delhi/CAS	8	Met306Val	WT	WT	WT
22	ASTR22/14	India	Euro-American superlineage	8	Met306Ile Glu378Ala	WT	WT	WT
23	ASTR23/14	India	Beijing	64	Met306Val	WT	Asp397Gly	WT
24	ASTR25/14	India	Beijing	4	Met306Ile	WT	Asp397Gly Ala83Thr	Arg223Arg
25	ASTR26/14	India	Delhi/CAS	–	Met306Ile	WT	WT	WT
26	ASTR27/15	India	Delhi/CAS	–	Met306Ile	WT	WT	WT
27	ASTR28/15	India	Beijing	–	Met306Val Asp328Gly	WT	Asp397Gly Leu201Leu	WT
28	ASTR29/15	India	Delhi/CAS	8	Met306Ile	WT	WT	WT
29	TDR32	Bangladesh	SCG2	16	Met306Ile	WT	Asp397Gly	–
30	TDR67	Rwanda	SCG5	32	Met306Val	WT	WT	–
31	TDR77	Korea	SCG2	4	WT	WT	Asp397Gly	–
32	TDR86	Korea	SCG2	4	WT	WT	Asp397Gly	–
33	TDR113	Korea	SCG2	16	Met306Val	WT	Asp397Gly	–
34	TDR114	Rwanda	SCG5	32	Met306Val	WT	WT	–
35	TDR115	Rwanda	SCG6b	32	Met306Val	WT	WT	–
36	TDR124	Korea	SCG2	32	Gln497Arg	WT	Asp397Gly	–
37	TDR132	Brazil	SCG5	16	Met306Ile	WT	WT	–
38	TDR151	Rwanda	SCG5	32	Met306Val	WT	WT	–

[a] Lineage of the *M. tuberculosis* isolates from India was determined by whole genome sequencing and of the TDR by SNP cluster grouping.
[b] Details given in References [4,11,13].

EAI lineage. Beijing lineage was observed in 8/49 (16.3%), Euro-American in 4/49 (8.2%), Haarlem in 3/49 (6%) and LAM in 1/49 (2%) isolates. One isolate showed a low coverage and the lineage could not be determined. Similar to the TDR isolates, the Indian isolates also revealed a strong association between *aftB* Asp397Gly mutation and the Beijing clade with 8/8 (100%) of Beijing isolates carrying the *aftB* Asp397Gly mutation.

4. Discussion

Arabinogalactan polymerization occurs through the action of a series of arabinan-transferases including the EmbCAB operon, aftA, aftB and aftC. Although, mutation analysis of *embC* (*Rv3793*), *embA* (*Rv3794*), and *embB* (*Rv3795*) genes show a moderate correlation (50–60%) between genotypic polymorphisms and phenotypic EMB resistance [29–32], various studies have also found many of these polymorphisms in EMB susceptible clinical isolates in addition to EMB resistant isolates [33,34]. The predominant determinants of EMB resistance are restricted to mutations in *embB*, e.g. *embB*306, *embB*406, *embB*497. These have been validated by either allelic exchange studies or overexpression studies in *M. smegmatis* and *M. tuberculosis* [5,7,32,35]. However, 35–40% of EMB resistant clinical isolates have no mutation in *embB* [27,29,30]. We had previously analysed mutations associated with EMB resistance in the *embB*, *embC*, *embA* and *Rv3806c* (*ubiA*) genes in EMB resistant and EMB susceptible clinical isolates of *M. tuberculosis* [13]. Mutations in *embB* were most common at codon 306 (ATG to ATC/GTG), occurring only in EMB resistant isolates (20/29; 69%). Mutations in the upstream region of *embA* at −8, −11, −12 and −60 codons also occurred in EMB resistant strains (8/29; 27.6%) of which 6/8 (75%) were observed in isolates with EMB MIC ≥16 μg/ml. However, no polymorphism significantly associated with EMB resistance was observed in *embC* or *ubiA* [13].

In the present study, we analysed polymorphisms in additional genes of the arabinogalactan-lipoarabinomannan pathway viz. *aftA*, *aftB* and *aftC* in isolates from India, Azerbaijan, Bangladesh, Belgium, Brazil, Cameroon, Korea and Rwanda by Sanger sequencing and whole genome sequencing.

Polymorphism analysis of the *aftA* and *aftC* genes in the two data sets did not reveal any mutation significantly associated with EMB resistance. Also, the analysis did not find any mutation in the predicted promoter (or the upstream region) of *aftA*, *aftB* and *aftC*.

Notably, analysis of the gene *aftB* demonstrated the presence of the

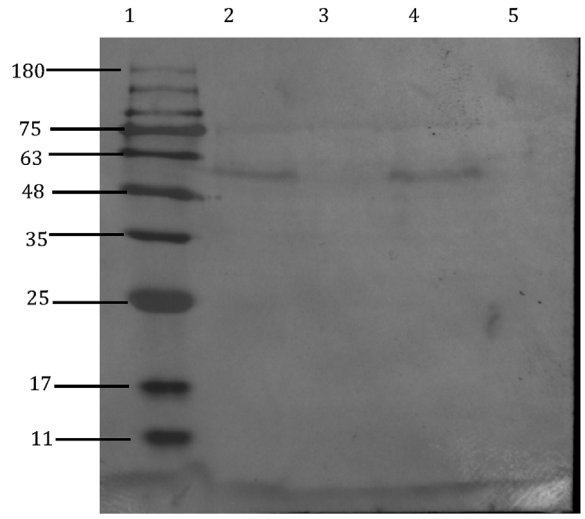

Fig. 1. Western blot analysis of *M. tuberculosis* H37Rv, overexpressing wild type (WT) *aftB* (↑*aftB*-WT) and mutant *aftB* (↑*aftB*-Mut). The overexpression strains were constructed using the mycobacterial shuttle vector pST-K. The *aftB* gene containing the Asp397Gly mutation (*aftB*-Mut) was amplified from the clinical isolate ASTR13/13. The WT gene (*aftB*-WT) was amplified from the laboratory strain H37Rv. lane 1: Protein marker; lane 2: overexpression of *aftB* in ↑*aftB*-WT; lane 4: overexpression of *aftB* in ↑*aftB*-Mut; lanes 3 and 5: H37Rv:pST-K:Nil (vector without *aftB* gene).

mutation Asp397Gly in 12/38 (31.6%) EMB resistant and 3/34 (8.8%) EMB susceptible Indian and TDR isolates (p 0.0216; Fisher exact test).

Of the twelve EMB resistant isolates with the *aftB* mutation Asp397Gly, 8/12 (66.7%) also had the canonical mutation Met306Val/Ile at *embB* and 1/12 (8.3%) each had the canonical mutations Leu402Val and Gln497Arg at *embB*. Though MIC was not determined for one of the twelve isolates, of the remaining isolates, eight showed high level resistance to EMB. Two isolates from Korea carried the Asp397Gly mutation at *aftB*, but did not have a mutation at *embB*. Interestingly, both these isolates had low level EMB resistance. Though the mutation Asp397Gly was also present in 3/34 (8.8%) EMB susceptible isolates, the difference between the presence of this mutation in EMB resistant and susceptible isolates was statistically significant. Based on the SNP analysis, it was natural to assume that the mutation Asp397Gly was associated with EMB resistance. Although *aftB* encodes for an arabinofuranosyl transferase similar to *embB*, Seidel et al. on the basis of their experiments on *Corynebacterium glutamicum* observed that *aftB* is not a target for EMB in *M. tuberculosis* [14]. However, *aftB* is present upstream to *ubiA* and helps in polymerization of arabinan moieties to the growing arabinogalactan chain and may thus have an indirect effect on EMB resistance mechanisms.

Hence, we adopted the overexpression approach to test the significance of Asp397Gly mutation with respect to EMB resistance. *aftB* carrying the Asp397Gly polymorphism was amplified from an EMB high level resistant isolate (ASTR13/13) with MIC 32 μg/ml. This isolate also harboured the canonical mutation *embB* Met306Val. To serve as a control, the WT *aftB* was amplified from *M. tuberculosis* H37Rv. Both the mutant as well as the WT genes were cloned in the shuttle vector pST-K and electroporated in H37Rv. MIC for EMB was subsequently tested for the recombinant strains as well as *M. tuberculosis* H37Rv. No change in the MIC of EMB was observed in ↑*aftB*-Mut when compared to ↑*aftB*-WT or *M. tuberculosis* H37Rv. The MIC of all the strains was found to be 1 μg/ml, which was lower than the Epidemiologic cut-off (ECOFF) for EMB [36] and was thus present in the susceptible range. This observation confirmed that *aftB* Asp397Gly was not responsible for EMB resistance.

To further confirm our findings, we correlated the genotype of the isolates with the presence or absence of the Asp397Gly mutation at *aftB*. Interestingly, all the 15 EMB resistant and susceptible isolates with the Asp397Gly mutation belonged to Beijing genotype, determined by whole genome sequencing; or to SCG2, determined by the SCG assay. The SCG2 genotype has been found to be concordant with the Beijing clade in earlier studies on *M. tuberculosis* [22,37,38].

In the present study, the occurrence of *aftB* Asp397Gly in Beijing isolates can be considered as a phylogenetic association with the lineage rather than resistance to EMB. The presence of this mutation mostly in the EMB resistant strains in the present study might be a consequence of smaller sample size, wherein not enough EMB susceptible isolates were tested for this polymorphism.

To conclude, we confirmed that Asp397Gly mutation in *aftB* lacked an association with EMB resistance indicating the need for further studies to find additional mechanisms of EMB resistance. However, in this study, we discovered and thus speculate that the *aftB* Asp397Gly mutation is in fact a molecular marker for the Beijing clade. The study also underscores the importance of a detailed analysis of clinical isolates before assigning a role to a novel mutation in drug resistance studies and highlights the effectiveness of whole genome sequencing for this purpose.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.tube.2019.01.004.

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