Mutation EthA\textsubscript{W21R} confers co-resistance to prothionamide and ethionamide in both Mycobacterium bovis BCG and Mycobacterium tuberculosis H37Rv

Abstract: Ethionamide (ETA) and prothionamide (PRO) are interchangeably used in tuberculosis (TB) chemotherapy regimens. Subtle discrepancies between biochemical and genetic information on the modes of sensitivity and resistance of isoniazid (INH) and ETA warrants further studies. We report a new mutation – EthA\textsubscript{W21R} – in Mycobacterium bovis Bacillus Calmette-Guérin that corresponds with co-resistance to both PRO and ETA, which to the best of our knowledge has not been reported before. Our findings suggest that mutation EthA\textsubscript{W21R} could be used as a marker site for testing PRO and ETA cross-resistance.

Keywords: mutation, EthA\textsubscript{W21R}, isoniazid, co-resistance, thioamides, molecular marker

The thioamide, ethionamide (ETA), and its propyl-analog prothionamide (PRO) are interchangeably used in tuberculosis (TB) chemotherapy regimens to treat multidrug-resistant TB (MDR-TB),\textsuperscript{1–3} drug-susceptible TB meningitis (TBM), and miliary TB in some settings, due to their good cerebrospinal fluid (CSF) penetration ability.\textsuperscript{4}

PRO is associated with better tolerance compared with ETA in the treatment of MDR-TB, and both are structurally similar to isoniazid (INH).\textsuperscript{2,5,6} The only notable distinction in their mechanism(s) of action is the lack of cross-resistance to INH.\textsuperscript{7,8}

Both ETA and PRO are prodrugs whose enzymatic activation by Mycobacterium tuberculosis' EthA inhibits InhA, which subsequently inhibits the M. tuberculosis' mycolic acid synthesis (Figure 1).\textsuperscript{1,9} Mutations in the ethA gene often underlie ETA and PRO mono-resistance.\textsuperscript{2} Hanouolle et al\textsuperscript{10} postulated that both are further transformed by EthA enzyme to a metabolite that accumulates intracellularly and acts as the final toxic compound. As illustrated in Figure 1,\textsuperscript{11} activated ETA and PRO form adducts with nicotinamide adenine dinucleotide (NAD), which is the inhibitor of the InhA enzyme in M. tuberculosis.\textsuperscript{1,12,13} Thee et al\textsuperscript{2} suggested that the correlation between mutations conferring ETA resistance and the MIC warrants further studies because of the subtle discrepancies between biochemical and genetic information on the modes of sensitivity and resistance in the cases of INH and ETA.

Here, we report a new mutation – EthA\textsubscript{W21R} – in Mycobacterium bovis Bacillus Calmette-Guérin (BCG) that corresponds with co-resistance to PRO and ETA, which to the best of our knowledge has not been reported before.

We screened wild-type M. bovis BCG Tice on high PRO concentrations and obtained one drug-resistant colony at 30 µg/mL PRO-containing 7H11 plate. To confirm the
Phenotypic resistance of the single colony, we similarly re-tested it on 30 and 40 µg/mL PRO-containing 7H11 plate. We sequenced the six reported genes (ethR, ethA, inhA, katG, ndh, and ahpC; Table 1; BGI, Shenzhen, China) associated with ETA and PRO resistance and found a single-nucleotide mutation in ethA gene leading to W21R mutation while the other five genes had no mutation(s).

We then overexpressed this mutated 1.4kb ethA<sub>W21R</sub> and the M. bovis ethA<sub>wt</sub> genes by cloning them at the NdeI and HindIII sites of extrachromosomal p60LuxN plasmid bearing the M. tuberculosis hsp60 promoter (Figure 2).<sup>14</sup> Recombinant plasmids p60<sub>ethA</sub>W21R and p60<sub>ethA</sub>wt constructs were verified by enzyme digestion and sequencing (BGI). Wild-type M. bovis BCG Tice and M. tuberculosis H37Rv strains were transformed with the plasmids p60<sub>ethA</sub>W21R and p60<sub>ethA</sub>wt through electroporation as described previously with some modifications.<sup>15</sup> Positive selection was confirmed by PCR amplification of the hygromycin resistance marker gene (hyg) in p60<sub>ethA</sub>W21R and p60<sub>ethA</sub>wt using primers hyg-r and hyg-f (Table 1).

We then evaluated the MICs of PRO and ETA against the recombinant and parental strains (control) using the classical agar plate method.<sup>16</sup> We show that after overexpressing the mutated ethA<sub>W21R</sub> in wild-type BCG and M. tuberculosis H37Rv, both PRO and ETA MIC rose by 256- and 128-fold, respectively (Table 2). Additionally, no observable differences were noted in the MICs of the overexpressed ethA<sub>wt</sub> recombinants and the parent strains (MIC = 0.25 and 0.5 µg/mL; Table 2). Our findings suggest that the mutation ethA<sub>W21R</sub> could be used as a marker site for testing PRO and ETA cross-resistance.

**Acknowledgments**

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EthA\textsubscript{W21R} confers co-resistance to thioamides

**Figure 2** E. coli–mycobacteria shuttle plasmids p60eth\textsubscript{AMt/wt}.

**Notes:** OriE, origin of replication region in E. coli; OriM, origin of replication in mycobacteria; hyg, hygromycin-resistant gene; ethA, ethA\textsuperscript{Wt} or ethA\textsuperscript{W21R}.

**Abbreviation:** E. coli, Escherichia coli.

**Table 1** PCR and sequencing primers used to delineate target-based spontaneous genotypic resistance mechanisms of M. bovis BCG

<table>
<thead>
<tr>
<th>Resistance to</th>
<th>Primer pairs</th>
<th>Nucleotide sequences (5′–3′)</th>
<th>Upstream extension (base)</th>
<th>Downstream extension (base)</th>
<th>Product length (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PRO EthRf/EthRr5</td>
<td>TTTTCCAGGATGCGGTAGC/CCGACGGATCTGAACA</td>
<td>185</td>
<td>263</td>
<td>1099</td>
<td></td>
</tr>
<tr>
<td>EthAf/EthAr</td>
<td>CCTGGCAGCTTACTACGTGTC/CGGCATCATCGTCGTCTG</td>
<td>75</td>
<td>54</td>
<td>1599</td>
<td></td>
</tr>
<tr>
<td>inhAf/inhAr</td>
<td>TCACGGCGGTAGAAGAGCA/CCACGCAGATGTCGCAAAGA</td>
<td>548</td>
<td>326</td>
<td>1684</td>
<td></td>
</tr>
<tr>
<td>KatGf/KatGr</td>
<td>TGCCAAAGATCACAACCTCA/AGACGACGGGTAGGCAAT</td>
<td>276</td>
<td>317</td>
<td>2816</td>
<td></td>
</tr>
<tr>
<td>Ndhf/Ndhr</td>
<td>ACTTGGCTCCGACGGCTAT/ATCCGGCGACC CCTCA</td>
<td>217</td>
<td>109</td>
<td>1718</td>
<td></td>
</tr>
<tr>
<td>ahpCf/ahpCr</td>
<td>CGACTGGCTCATATCGGCAGAAT/AAATACCTGCGGATTCGTT</td>
<td>216</td>
<td>180</td>
<td>984</td>
<td></td>
</tr>
</tbody>
</table>

**EthAf2**

GGAAATCCGATATGACCAGCACCTGACGTT

**EthAr2**

CCCAAGCTTCTAAACCCCCACGGGGCA

**hyg-f**

GTGACACAAAGATCCCTG

**hyg-r**

TCAGGCGCCGGGCGGCGT

**Note:** Primers for each gene amplification were extended with ~150 bp upstream and downstream of start and stop codons.

**Abbreviations:** M. bovis, Mycobacterium bovis; PRO, prothionamide.

**Table 2** MICs of PRO and ETA for wild-type and recombinant strains

<table>
<thead>
<tr>
<th>Serial number</th>
<th>Strain</th>
<th>Mutations</th>
<th>MICs (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>PRO</td>
</tr>
<tr>
<td>1</td>
<td>M. tuberculosis H37Rv:p60eth\textsubscript{Am}</td>
<td>W21R</td>
<td>32</td>
</tr>
<tr>
<td>2</td>
<td>M. tuberculosis H37Rv:p60eth\textsubscript{Am}</td>
<td>–</td>
<td>0.25</td>
</tr>
<tr>
<td>3</td>
<td>M. tuberculosis H37Rv Wt</td>
<td>–</td>
<td>0.5</td>
</tr>
<tr>
<td>4</td>
<td>M. bovis BCG Tice BCG:p60eth\textsubscript{Am}</td>
<td>W21R</td>
<td>32</td>
</tr>
<tr>
<td>5</td>
<td>M. bovis BCG Tice BCG:p60eth\textsubscript{Am}</td>
<td>–</td>
<td>0.5</td>
</tr>
<tr>
<td>6</td>
<td>M. bovis BCG Tice Wt</td>
<td>–</td>
<td>0.25</td>
</tr>
</tbody>
</table>

**Abbreviations:** ETA, ethionamide; M. bovis, Mycobacterium bovis; M. tuberculosis, Mycobacterium tuberculosis; PRO, prothionamide.
China: 1) State Key Laboratory of Respiratory Disease, Guangzhou Institutes of Biomedicine and Health, Chinese Academy of Sciences, Guangzhou, China, and 2) State Key Laboratory of Respiratory Disease, Department of Clinical Laboratory, Guangzhou Chest Hospital, Guangzhou, China. The facilities are compliant with biosafety level 2+ and 3 requirements for handling infectious materials.

**Disclosure**
The authors report no conflicts of interest in this work.

**References**