Article

How a PCR Sequencing Strategy Can Bring New Data to Improve the Diagnosis of Ethionamide Resistance

Thomas Maitre 1,2,3,†, Florence Morel 1,2,†, Florence Brossier 1,2,‡, Wladimir Sougakoff 1,2,‡, Jérémy Jaffre 1,2, Sokleaph Cheng 1, Nicolas Veziris 1,2,§, Alexandra Aubry 1,2,‡ and on behalf of the NRC-MyRMA 8

1 Centre National de Référence des Mycobactéries et de la Résistance des Mycobactéries aux Antituberculeux, Hôpital Pitié-Salpêtrière, AP-HP (Assistance Publique Hôpitaux de Paris), Sorbonne-Université, F-75013 Paris, France; thomas.maitre@aphp.fr (T.M.); florence.morel2@aphp.fr (F.M.); florence.brossier@aphp.fr (F.B.); wladimir.sougakoff@aphp.fr (W.S.); jeremy.jaffre@aphp.fr (J.J.); sokleaph.cheng@aphp.fr (S.C.); nicolas.veziris@sorbonne-universite.fr (N.V.)
2 Centre d’Immunologie et des Maladies Infectieuses, Sorbonne Université, INSERM, U1135, Cimi-Paris, F-75013 Paris, France
3 Department of Pneumology and Thoracic Oncology, Reference Centre for Rare Lung Diseases, Tenon Hospital, AP-HP, F-75020 Paris, France
4 Département de Bactériologie, AP-HP, Hôpital Saint-Antoine, Sorbonne-Université, F-75012 Paris, France
* Correspondence: alexandra.aubry@sorbonne-universite.fr; Tel.: +33-1-40-77-97-68
† These authors contributed equally to this work.
‡ Deceased.
§ The members of the CNR MyRMA (French National Reference Center for Mycobacteria) are: Isabelle Bonnet, Emmanuelle Cambau, Faiza Mougari, Vichita Ok, Jerôme Robert.

Abstract: Ethionamide (ETH) is a second-line antituberculosis drug. ETH resistance (ETH-R) is mainly related to the mutations of the monoxygenase-activating ETH (EthA), the ETH target (InhA), and the inhA promoter. Nonetheless, diagnosing ETH-R is still challenging. We assessed the strategy used for detecting ETH-R at the French National Reference Center for Mycobacteria in 497 MDR-TB isolates received from 2008 to 2016. The genotypic ETH’s resistance detection was performed by sequencing ethA, ethR, the ethA-ethR intergenic region, and the inhA promoter in the 497 multidrug-resistant isolates, whereas the phenotypic ETH susceptibility testing (PST) was performed using the reference proportion method. Mutations were found in up to 76% of the 387 resistant isolates and in up to 28% of the 110 susceptible isolates. Our results do not support the role of ethR mutations in ETH resistance. Altogether, the positive predictive value of our genotypic strategy to diagnose ETH-R was improved when only considering the variants included in the WHO catalogue and in other databases, such as TB-Profiler. Therefore, our work will help to update the list of mutations that could be graded as being associated with resistance to improve ETH-R diagnosis.

Keywords: ethionamide; tuberculosis; resistance; molecular diagnosis

1. Introduction

The emergence of multidrug-resistant tuberculosis (MDR-TB), i.e., resistant to at least rifampin and isoniazid (INH), further threatens TB control worldwide [1]. MDR-TB remains challenging to treat, requiring second-line anti-TB drugs such as ethionamide (ETH).

Over the past several years, molecular techniques have been developed for the rapid detection of resistance to antituberculous agents since the quick detection of drug resistance is crucial for designing appropriate antituberculosis drug regimens, preventing treatment failure and/or relapse, and reducing the spread of drug-resistant isolates. Molecular assays for the detection of mutations related to resistance have been increasingly used and have led to shortening the time to detection of resistance to one working day [2].

Ethionamide (ETH) is a derivative of isonicotinic acid that is structurally similar to INH. ETH and INH are pro-drugs requiring activation by different pathways: the KatG
catalase-peroxidase for INH and the EthA monooxygenase (negatively regulated by EthR) for ETH [3]. ETH and INH have a common target: the enoyl-ACP reductase InhA. EthA is a NADPH-specific flavin adenine dinucleotide-containing monooxygenase and a Baeyer–Villiger monooxygenase and is involved in cell wall biosynthesis [4,5]. Previous studies have reported that mutations of ethA and inhA are the main mutations reported in ETH-R strains: ethA mutations caused 37% to 100% of ETH-R in M. tuberculosis, and mutations in inhA caused 25% to 100% of ETH-R in M. tuberculosis, whereas mutations in EthR and the inhA promoter were less frequent [6,7].

The increasing use of genotypic diagnosis of resistance in tuberculosis management requires the extensive study and classification of mutations identified in genes involved in drug resistance, i.e., genotypic/phenotypic correlation. For ETH, only 24 mutations were confidently graded by the WHO as being associated with resistance in ethA, inhA, and the promoter region of inhA, which stresses the need to add new data enabling an update to that list (without taking into account the mutations graded as “uncertain significance” that are listed in the extended catalogue [8]).

Therefore, the present study aimed to investigate the prevalence of mutations in ethA, ethR, the inhA gene, and the promoter region of inhA associated with independent resistance to ethionamide (ETH-R) in M. tuberculosis isolates based on a prospective genotyping strategy used at the French National Reference Center (NRC) for Mycobacteria to diagnose ETH-R.

2. Materials and Methods
2.1. Mycobacterium tuberculosis Complex Clinical Isolates

A total of 497 MDR M. tuberculosis complex isolates from TB cases diagnosed in France and received at the French National Reference Center for Mycobacteria were collected over a 9-year (2008–2016) period. On the basis of the results of DST, the 497 MDR isolates were classified as ETH-R (n = 387) or as susceptible to ETH (ETH-S) (n = 110) (Table 1).

Table 1. Mutations in ethA, inhA and its promoter, ethR, and ethA-ethR intergenic region for the 387 ETH-R MDR-TB isolates.

| N° of Isolates | Sequencing Results a | ethA Promoter | inhA | ethR | ethA-ethR Intergenic Region |
|---------------|----------------------|---------------|------|------|-----------------------------|
| 1             | M1R                  | wt            | np   | wt   | wt                          |
| 2             | G11S                 | wt            | np   | wt   | wt                          |
| 2             | S15P                 | c-15t         | np   | wt   | wt                          |
| 1             | A19V                 | g-17t         | np   | wt   | wt                          |
| 1             | H22P                 | wt            | np   | wt   | wt                          |
| 1             | H22P                 | c-15t         | np   | wt   | wt                          |
| 3             | H22P                 | c-15t         | np   | F110L | wt                          |
| 1             | C27W                 | wt            | np   | wt   | wt                          |
| 1             | C27W                 | g-17t         | np   | wt   | wt                          |
| 1             | G36D                 | wt            | np   | wt   | wt                          |
| 1             | G42V, P334A          | wt            | np   | wt   | wt                          |
| 1             | F48S                 | wt            | np   | wt   | wt                          |
| 1             | Y50C                 | wt            | np   | wt   | wt                          |
| 1             | S35C                 | wt            | np   | wt   | wt                          |
| 1             | F66L, G299D          | wt            | np   | wt   | wt                          |
| 1             | G78D                 | c-15t         | np   | wt   | wt                          |
| 1             | A89E, S266R          | wt            | np   | wt   | wt                          |
| 1             | A89E, R99Q, S266R    | wt            | np   | wt   | a-9g                        |
| 1             | D95N                 | wt            | np   | wt   | wt                          |
| 1             | D95N                 | c-15t         | S94A | wt   | wt                          |
| 1             | D95N                 | c-15t         | np   | wt   | wt                          |
### Table 1. Cont.

| No. of Isolates | Sequencing Results * |   |
|-----------------|-----------------------|---|
|                 | ethA                  | inhA Promoter | inhA | ethR | ethA-ethR |
|                 |                       |               |      |      | Intergenic Region |
| 1               | W109 !                | wt            | np   | wt   | wt        |
| 1               | G124D                 | wt            | np   | wt   | wt        |
| 2               | L136R                 | B-8a          | np   | wt   | wt        |
| 2               | C137R                 | wt            | np   | wt   | wt        |
| 1               | G139D                 | wt            | np   | wt   | wt        |
| 2               | Y140 !                | wt            | np   | wt   | wt        |
| 1               | Y141C, 1367_ins_7nt   | wt            | np   | M142I, Q143K | wt  |
| 1               | Y147 !                | wt            | np   | wt   | wt        |
| 6               | Q165P                 | wt            | np   | wt   | wt        |
| 2               | W167G                 | wt            | np   | wt   | wt        |
| 1               | S183R                 | C-15t         | np   | wt   | wt        |
| 1               | P192S                 | C-15t         | np   | wt   | wt        |
| 1               | P192T                 | wt            | np   | wt   | wt        |
| 1               | V202G                 | wt            | np   | wt   | wt        |
| 1               | Q206 !                | wt            | np   | wt   | wt        |
| 2               | S208 !                | wt            | np   | wt   | wt        |
| 1               | Y211S                 | C-15t         | np   | wt   | wt        |
| 1               | E225K                 | C-15t         | np   | wt   | wt        |
| 1               | N226D                 | wt            | np   | wt   | wt        |
| 3               | V238G                 | wt            | np   | wt   | wt        |
| 1               | R239L                 | wt            | np   | wt   | wt        |
| 1               | Q254P                 | C-15t         | np   | wt   | wt        |
| 1               | W256 !                | wt            | np   | wt   | wt        |
| 4               | P257S                 | C-15t         | np   | wt   | wt        |
| 3               | S266R                 | wt            | np   | wt   | wt        |
| 9               | Q269 !                | C-15t         | np   | wt   | wt        |
| 1               | Q269 !                | C-15t         | np   | wt   | a-9g      |
| 1               | L272P                 | wt            | np   | wt   | wt        |
| 4               | H281P                 | C-15t         | np   | wt   | wt        |
| 1               | C294Y                 | C-15t         | np   | wt   | wt        |
| 1               | I305N                 | wt            | np   | wt   | wt        |
| 2               | T314I                 | wt            | np   | wt   | wt        |
| 1               | T314I                 | C-15t         | np   | wt   | wt        |
| 1               | T314I                 | T-8c          | np   | wt   | wt        |
| 1               | T314I                 | T-8c          | np   | wt   | wt        |
| 1               | I337V                 | WT            | V78A | wt   | wt        |
| 3               | I338S                 | C-15t         | np   | wt   | wt        |
| 1               | T342K                 | wt            | np   | wt   | wt        |
| 1               | M372R                 | wt            | np   | wt   | wt        |
| 2               | N379D                 | wt            | np   | wt   | wt        |
| 1               | G385D                 | wt            | np   | wt   | wt        |
| 2               | C403R                 | wt            | np   | wt   | wt        |
| 2               | P422L                 | wt            | np   | wt   | wt        |
| 2               | L440P                 | wt            | np   | wt   | wt        |
| 1               | Q449R                 | wt            | np   | wt   | wt        |
| 1               | D464G                 | wt            | np   | wt   | wt        |
| 1               | R471P                 | C-15t         | np   | wt   | wt        |
| 1               | R483T                 | wt            | np   | wt   | wt        |
| 1               | 32_del_g              | wt            | np   | wt   | wt        |
| 1               | 57_ins_4nt            | T-8c          | np   | wt   | wt        |
| 1               | 109_del_a             | T-8c          | np   | wt   | wt        |
| 19              | 110_del_a             | wt            | np   | wt   | wt        |
| 1               | 110_del_a             | T-8c          | np   | wt   | wt        |
| 1               | 137_del_a             | T-8c          | np   | wt   | wt        |
| 1               | 328_ins_t             | wt            | np   | wt   | wt        |
Table 1. Cont.

| N° of Isolates | Sequencing Results a |
|----------------|----------------------|
|                | ethA | inhA Promoter | inhA | ethR | ethA-ethR Intergenic Region |
| 1              | 373_ins_a | c-15t | np | wt | wt |
| 1              | 390_del_c | wt | np | wt | wt |
| 1              | 437_ins_g | wt | np | wt | wt |
| 1              | 477_del_g | wt | np | wt | wt |
| 1              | 509_del_a | wt | np | M102T | wt |
| 1              | 522_del_c | wt | np | wt | wt |
| 1              | 537–790_del | wt | np | wt | wt |
| 1              | 626_del_cc | wt | np | wt | wt |
| 3              | 639_del_gt | wt | np | wt | wt |
| 5              | 703_del_t | wt | np | wt | wt |
| 1              | 751_del_a | wt | np | wt | wt |
| 1              | 752_ins_g | wt | np | wt | wt |
| 9              | 768_del_g | wt | np | wt | wt |
| 1              | 778_del_a | c-15t | np | wt | wt |
| 2              | 831–837_del | c-15t | np | wt | wt |
| 4              | 884_del_t | wt | np | wt | wt |
| 1              | 935_ins_t | wt | np | wt | wt |
| 1              | 1010_del_t | wt | np | wt | wt |
| 1              | 1034_del_a | wt | np | wt | wt |
| 1              | 1054_del_g | t-8c | np | wt | wt |
| 1              | 1061_ins_c | c-15t | np | wt | wt |
| 1              | 1222_del_t | wt | np | wt | wt |
| 6              | 1242_del_t | wt | np | wt | wt |
| 1              | 1281_ins_a | wt | np | wt | wt |
| 1              | 1292_del_t | wt | np | wt | wt |
| 1              | 1292_del_t | wt | np | wt | a-9g |
| 1              | 1343_del_a | c-15t | np | wt | wt |
| 1              | 1391_ins_a | c-15t | np | wt | wt |
| 1              | 1431_ins_t | WT | np | wt | wt |
| 1              | 1466_del_tt | c-15t | np | wt | wt |
| 5              | 1470_del_g | wt | np | wt | wt |
| 1              | 933–1737_del | wt | np | wt | a-40g |
| 3              | large deletion | wt | np | large deletion | large deletion |
| 1              | large deletion | wt | np | wt | wt |
| 1              | large deletion | wt | np | wt | wt |
| 1              | large deletion | wt | np | wt | wt |
| 8              | wt | wt | S94A | wt | wt |
| 1              | wt | wt | S94A | wt | wt |
| 1              | wt | wt | S94A | wt | wt |
| 82             | wt | wt | wt | T149A | wt |
| 1              | wt | wt | wt | S131R | wt |
| 1              | wt | wt | wt | M142I, Q143K | wt |
| 1              | wt | wt | wt | P195L | wt |
| 93             | wt | c-15t | np | wt | wt |

Mutations are indicated as amino acids for all proteins encoded by the corresponding genes except for the inhA promoter and the ethA-ethR intergenic region, for which mutations are indicated in nucleotide. Mutations reported in the WHO catalogue as being associated with ETH-R are bolded and underlined. Mutations not listed in the WHO catalogue but mentioned in other published databases are bolded. Phylogenetic SNPs are indicated in italics. Stop codon is represented with “!”.

2.2. Phenotypic Drug Susceptibility Testing and Quality Controls

DST was performed on Löwenstein–Jensen medium using the reference standard proportion method [9] and concentrations of 40 mg/L for ETH [10,11]. Resistance to ETH was defined as a proportion of resistant mutants ≥ 1% at 40 mg/L [10,11]. Quality controls were performed for each new batch of LJ medium containing ETH using the reference
strain *M. tuberculosis* H37Rv. The laboratory underwent external quality validation through the External Quality Assurance (EQA) systems, ensuring the accurate diagnosis of TB and drug-resistant TB through the European TB reference laboratory network (ERLTB-Net) organized by INSTAND.

2.3. DNA Sequencing of Genes Associated with Ethionamide Resistance

For all of the MDR-TB isolates received at the French NRC, the entire *ethA* and *ethR* genes, and the *ethA-ethR* intergenic region were prospectively sequenced, and mutations in the *inhA* promoter region were determined by using the Genotype MTBDRplus test (Hain Lifescience GmbH, Nehren, Germany) according to the manufacturer’s instructions. For the isolates in which wild-type *ethA* and *inhA* promoter were observed, the entire *inhA* gene and its promoter were retrospectively sequenced in ETH-R isolates. *EthA*, *ethR*, the *ethA-ethR* intergenic region, and *inhA* and its promoter were amplified and sequenced using the primers previously described [6]. Genomic DNA was isolated from bacteria grown on Lowenstein–Jensen medium. A loop of culture was resuspended in water (500 µL) and inactivated by heating at 95 °C for 15 min. DNA (5 µL) obtained by heat shock extraction (1 min at 95 °C and 1 min in ice, repeated five times) was used for PCR amplification with the following steps: denaturation of 5 min at 95 °C followed by 35 cycles of 1 min at 95 °C, 1 min at the primer-dependent annealing temperature (Tm), 1 min at 72 °C, and a final extension step of 7 min at 72 °C. The PCR products were purified by filtration with Microcon 100 microconcentrators (Amicon Inc., Beverly, MA, USA), and the PCR for Sanger sequencing was conducted using a BigDye Terminator cycle sequencing ready kit (Applied Biosystems, Courtaboeuf, France).

2.4. Databases

The impact of the mutations on ETH-R was evaluated by looking for those graded as being associated with resistance in the WHO catalogue. For those not graded as being associated with ETH-R in the catalogue, we checked if they were listed in other published databases, such as TB-Profiler [12,13], PhyResSE [14], and the one published by Manson et al. [15].

2.5. Statistical Analysis

The proportion of the ETH-R and ETH-S isolates with the different mutations were compared using Fisher’s exact test. The *p* values were two-tailed, and *p* values of ≤0.05 were considered significant. Sensitivity, specificity, positive predictive value, and negative predictive value of the prospective genotypic strategy used at the French NRC were determined by considering the phenotypic DST as the reference standard. Since the entire *inhA* gene was only sequenced retrospectively in ETH-R isolates displaying wild-type *ethA* and the *inhA* promoter, it is not considered as part of the prospective genotypic strategy.

3. Results

Among the 497 MDR-TB isolates, 78% (*n* = 387) were classified as ETH-R and 22% (*n* = 110) as ETH-S by phenotypic DST (Tables 1 and 2). A total of 123 mutations were evidenced in *ethA*, *ethR*, the *ethA-ethR* intergenic region, and *inhA* and its promoter.

Most of the alterations observed had never been reported, no matter their susceptibility to ETH [6,16–19], and only 6 (5%) were cited as being associated with resistance in the WHO catalogue [20], and 20 additional mutations among the 123 (17%) were cited in published databases of drug resistance mutations (Tables 1 and 2). Altogether, among the 387 ETH-R isolates, 294 (76%) had at least one mutation in *ethA*, *ethR*, the *ethA-ethR* intergenic region, and *inhA* and its promoter. Most of the alterations observed had never been reported, no matter their susceptibility to ETH [6,16–19], and only 6 (5%) were cited as being associated with resistance in the WHO catalogue [20], and 20 additional mutations among the 123 (17%) were cited in published databases of drug resistance mutations (Tables 1 and 2). Altogether, among the 387 ETH-R isolates, 294 (76%) had at least one mutation in *ethA*, *ethR*, the *ethA-ethR* intergenic region, and *inhA* promoter, and 93 (24%) had no mutation, whereas among the 110 ETH-S isolates, 79 (72%) had no mutations in *ethA*, *ethR*, the *ethA-ethR* intergenic region, and the *inhA* promoter, and 31 (28%) had at least one mutation (*p* < 10^{-5}) (Table 1).
Table 2. Mutations in ethA, inhA and its promoter, ethR, and ethA-ethR intergenic region for the 110 ETH-S MDR-TB isolates.

| N° of Isolates | Sequencing Results a |
|----------------|----------------------|
|                | ethA | inhA | inhA | ethR | ethA-ethR |
|                | Promoter |       |       |       | Intergenic Region |
| 2              | I9T   | wt   | np   | wt   | wt         |
| 1              | G11D  | wt   | np   | wt   | wt         |
| 2              | D95N, 768_del_g | wt | np | wt | wt |
| 1              | C131Y | wt   | np   | wt   | wt         |
| 2              | W167S, S266R | wt | np | S131R | wt |
| 1              | II78S | wt   | np   | wt   | wt         |
| 3              | S266R | wt   | np   | wt   | wt         |
| 1              | C294Y | wt   | np   | wt   | wt         |
| 1              | T314I | wt   | np   | wt   | wt         |
| 1              | P334A | wt   | np   | wt   | wt         |
| 1              | N379D | wt   | np   | wt   | wt         |
| 1              | 110_del_a | wt | np | wt | wt |
| 1              | 382_ins_g | wt | np | wt | wt |
| 1              | 626_del_cc | wt | np | wt | wt |
| 3              | 703_del_t | wt | np | wt | wt |
| 2              | 768_del_g | wt | np | wt | wt |
| 1              | 851_ins_c | wt | np | wt | wt |
| 1              | 935_ins_t | wt | np | wt | wt |
| 1              | 1034_del_a | wt | np | wt | wt |
| 1              | 1242_del_t | wt | np | wt | wt |
| 1              | wt | wt | 65_ins_cg | wt | wt |
| 2              | wt | c-15t | np | wt | wt |
| 79             | wt | wt | np | wt | wt |

Mutations are indicated as amino acids for all proteins encoded by the corresponding genes except for the inhA promoter and the ethA-ethR intergenic region for which mutations are indicated in nucleotide. Mutations reported in the WHO catalogue as being associated with ETH-R are bolded and underlined. Mutations not listed in the WHO catalogue but mentioned in other published databases are bolded. Phylogenetic SNPs are indicated in italics. a wt: wild-type; mut: mutated, np: not performed.

Among the 123 mutations evidenced in ethA, ethR, the ethA/ethR intergenic region, and inhA and its promoter, 4 corresponded to phylogenetic SNPs: G124D, S266R, and 768_del_g in EthA and V78A in InhA (Table 3) [21], and therefore, they have no impact on ETH susceptibility.

Table 3. Performances of the sequencing strategy used to diagnose ETH-R (i.e., PCR sequencing of ethA, inhA and its promoter, ethR, and ethA-ethR intergenic region) regarding the criteria used to interpret the results, compared to the phenotypic DST as a gold standard.

| Criteria Used to Interpret the Mutations | Sensitivity | Specificity | PPV a | NPV b |
|-----------------------------------------|-------------|-------------|-------|-------|
| none c                                  | 78.6        | 71.8        | 90.7  | 48.8  |
| none, ethR mutations excluded           | 74.9        | 72.7        | 90.6  | 45.2  |
| none, polymorphisms excluded           | 77.8        | 76.4        | 92    | 49.4  |
| none, polymorphisms and ethR mutations excluded | 75.2        | 76.4        | 91.8  | 46.7  |
| WHO catalogue only                      | 36.7        | 98.2        | 98.6  | 30.6  |
| WHO catalogue + databases               | 53.2        | 98.2        | 99.0  | 37.4  |
| WHO catalogue + databases (ethR mutation excluded) | 52.9        | 98.2        | 99.0  | 37.2  |

a PPV: positive predictive value; b NPV: negative predictive value; c all mutations are taken into account.

Among the 123 mutations, 112 (91%) were only evidenced in the ETH-R strains. Among these 112 mutations, after the exclusion of known polymorphisms, 61 (54%) were
the only mutations evidenced, and no other mutation was found in the candidate genes. Among the 112 different mutations that were only observed in ETH-R strains, almost half (n = 57.5%) are reported as being associated with ETH-R in the WHO catalogue and/or other databases (Table 1).

Interestingly, the mutations suspected to have an important impact on protein function (i.e., the introduction of a stop codon and large deletions) were not evidenced at all in the ETH-S strains (Table 2).

By comparing the proportions of the mutated strains in the ETH-R and ETH-S strains per gene, the difference was statistically significant for ethA (197/387 (51%) versus 28/110 (25%), p < 10^{-5}), the inhA promoter (139/387 (36%) versus 2/110 (2%), p < 10^{-5}), and the ethA-ethR intergenic region (15/387 (4%) versus 0/110 (0%), p = 0.035), but not for ethR (13/387 (3%) versus 3/110 (3%), p = 0.74) (Tables 1 and 2).

By using the phenotypic data as the reference, the genotyping strategy used in the French National Reference Center (NRC) for Mycobacteria had 78.8% sensitivity to diagnose ETH-R, 76.4% specificity, 92.0% positive predictive value (PPV), and 49.4% negative predictive value (NPV) (with the exclusion of known phylogenetic SNPs). Modifying this strategy by deleting ethR mutations whose role in ETH-R is unclear [6,22,23] and did not modify its performance (Table 3), whereas only considering the mutations mentioned in the WHO catalogue dramatically increased the PPV performance [20]. Interestingly, when combining the interpretation of the impact of the mutations and the published databases to the WHO catalogue, the sensitivity increased dramatically (36.7% vs. 52.9%), but the PPV of the genotyping strategy used to predict ETH-R only slightly increased at the expense of the NPV value (Table 3).

4. Discussion

The old antibiotic ETH, which was used for a long time to treat drug-resistant TB [1], is benefiting from a renewal since the development of new compounds boosting its activity in vivo [24,25] are currently being assessed in a Phase 1 trial (ClinicalTrials.gov n° NCT04654143).

The increase in the proportion of ETH-R isolates among the MDR-TB isolates received at the French NRC from 2008–2009 (44%) to 2010–2016 (77%) represents a challenge to the existing health care facilities for the management of MDR-TB and XDR-TB who follow programmatic regimens, and this underscores the need for reliable methods for ETH DST [26].

The previously published studies aiming at deciphering the molecular bases of resistance to ETH [6,27–32] had some limitations: Firstly, most of the studies were performed on retrospectively chosen isolates, either ETH-R isolates exclusively or in a high prevalence of ETH-R isolates. Secondly, no study included the sequencing of all of the main genes described as being implicated in ETH-R (i.e., ethA, ethR, the ethA-ethR intergenic region, and inhA and its promoter). Therefore, our study prospectively evaluated the performance of a genotypic strategy based on the sequencing of the main genes known to be involved in ETH-R to diagnose ETH-R, which brings useful light into the field, especially as whole genome sequencing becomes widely used, making it even more complex to diagnose the resistance of a drug such as ETH.

Overall, the performance of our genotypic strategy to diagnose ETH-R had suboptimal performance (Table 3). Interestingly, the performance of our genotypic strategy was enhanced by combining the interpretation of the mutations based on the data from the WHO catalogue and other databases (Table 3), as illustrated by the improvement in the specificity (98.2% vs. 76.4%) and the PPV (99.0% vs. 92.0%). Since the role of ethR mutations in ETH-R is doubtful, we propose to avoid taking these mutations into account to diagnose ETH-R [6,22,23]. Our genotypic strategy, when either taking the ethR mutations into account or not, did not modify the diagnostic performance, confirming the probable lack of involvement in the resistance of ethR mutations (Table 3).
In light of these results, carefully analyzing the strains without genotypic/phenotypic correlation is of great interest. The 26 ETH-S isolates with at least one alteration, phylogenetic SNPs excluded (Table 2), may be explained by the fact that (i) the genetic alteration observed is not implicated in ETH-R, (ii) the strains have a low level of ETH-R which is not detected by the phenotypic DST [33–36], or (iii) compensatory mutations restore ETH susceptibility. Other flavin monooxygenases such as EthA2 or MymA are involved in ETH activation [24,37]. These alternative activation pathways may compensate for the impact of mutations in EthA that are suspected to provide a loss of protein function (i.e., deletion or insertion) by restoring susceptibility to ETH despite the presence of an EthA mutation (Table 2). It is worth noticing that no mutations that are suspected to provide an important loss of protein function (stop codon and large deletions) were observed in the ETH-S strains (Table 2). The 96 ETH-R isolates without any mutations (phylogenetic SNPs excluded) may be explained by the fact that (i) mutations are present in genes other than those studied (mshA, Rv3083, ndh, Rv0565c [38,39]), and (ii) the strains were wrongly classified as resistant by the phenotypic DST (see below).

Overall, both false results questioned the ability of genotyping, but also of phenotypic DST, to properly classify strains as susceptible or resistant. The challenges associated with \textit{M. tuberculosis} DST are well known, especially for ETH. First, the drug is thermolabile, which makes DST difficult [33]. Second, discriminating ETH-resistant and -susceptible strains can be a challenge since the distribution of their Minimum Inhibitory Concentrations (MICs) partially overlap [34]. This is similar to what has been described for ethambutol, supporting the idea that the reporting of strains with MICs close to the ECOFF could be affected by reproducibility issues, as the classification into susceptible or resistant highly depends on methodological variation [34]. Third, ETH DST has been shown to have poor concordance and reproducibility compared to other drugs [35,36]. As previously suggested, creating an intermediate susceptibility classification for ETH-S strains with gene alteration, supported by the unfavorable pharmacodynamic indices, could be warranted to increase reproducibility and to account for methodological variation [34].

Most of the alterations described in our study (about half of the mutations exclusively found in ETH-R strains) had never been described before, and little is currently known about the effects of the different mutations found in \textit{ethA}, \textit{ethR}, and the \textit{ethA-ethR} intergenic region. Therefore, we have provided new data that can contribute to enriching the listed mutations that are associated with ETH-R in the WHO catalogue and other databases.

Author Contributions: Conceptualization, F.B. and A.A.; methodology, T.M., F.M., F.B., W.S. and J.J.; software, T.M., F.M. and F.B.; validation, T.M., F.M., F.B., W.S., J.J., S.C., N.V. and A.A.; formal analysis, F.M., F.B., W.S. and A.A.; investigation, T.M., F.M., F.B., W.S., J.J., S.C., N.V. and A.A.; resources, N.V. and A.A.; data curation, T.M., F.M. and A.A.; writing—original draft preparation, T.M., F.M., W.S., F.B., N.V. and A.A.; writing—review and editing, T.M., F.M., J.J., S.C., N.V. and A.A.; visualization, T.M., F.M., F.B., W.S., J.J., S.C., N.V. and A.A.; supervision, A.A.; project administration, A.A.; funding acquisition, T.M., N.V. and A.A. All authors have read and agreed to the published version of the manuscript.

Funding: This work was supported by grants from the Institut National de la Santé et de la Recherche Médicale (INSERM), and annual grants from Santé Publique France and Sorbonne Université.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study are available on request from the corresponding author.

Acknowledgments: We wish to honor the memory of our colleagues, Florence Brossier and Wladimir Sougakoff. We thank all the technicians working at the French NRC for their dedication to this work.

Conflicts of Interest: The authors declare no conflict of interest.
References

1. World Health Organization WHO. Consolidated Guidelines on Drug-Resistant Tuberculosis Treatment; WHO: Geneva, Switzerland, 2019.

2. Brossier, F.; Sougakoff, W. French National Reference Center for Mycobacteria. Molecular Detection Methods of Resistance to Antituberculosis Drugs in Mycobacterium Tuberculosis. Med. Mal. Infect. 2017, 47, 340–348. [CrossRef] [PubMed]

3. Baulard, A.R.; Betts, J.C.; Engohang-Ndong, J.; Quan, S.; McAdam, R.A.; Brennan, P.J.; Locht, C.; Besra, G.S. Activation of the Pro-Drug Ethionamide Is Regulated in Mycobacteria. J. Biol. Chem. 2000, 275, 28326–28331. [CrossRef] [PubMed]

4. Dover, L.G.; Alahari, A.; Gratraud, P.; Gomes, J.M.; Bhowruth, V.; Reynolds, R.C.; Besra, G.S.; Kremer, L. EthA, a Common Activator of Thiocarbamide-Containing Drugs Acting on Different Mycobacterial Targets. Antimicrob. Agents Chemother. 2007, 51, 1055–1063. [CrossRef] [PubMed]

5. Quémard, A.; Lanéelle, G.; Lacave, C. Mycolic Acid Synthesis: A Target for Ethionamide in Mycobacteria? Antimicrob. Agents Chemother. 1992, 36, 1316–1321. [CrossRef] [PubMed]

6. Brossier, F.; Veziris, N.; Truffot-Pernot, C.; Jarlier, V.; Sougakoff, W. Molecular Investigation of Resistance to the Antituberculous Drug Ethionamide in Multidrug-Resistant Tuberculous Clinical Isolates of Mycobacterium Tuberculosis. Antimicrob. Agents Chemother. 2011, 55, 355–360. [CrossRef] [PubMed]

7. DeBarber, A.E.; Mdluli, K.; Bosman, M.; Bekker, L.G.; Barry, C.E. Ethionamide Activation and Sensitivity in Multidrug-Resistant Mycobacterium Tuberculosis. Proc. Natl. Acad. Sci. USA 2000, 97, 9677–9682. [CrossRef] [PubMed]

8. World Health Organization. Target Product Profile for Next-Generation Drug-Susceptibility Testing at Peripheral Centres; World Health Organization: Geneva, Switzerland, 2021; ISBN 978-92-4-003236-1.

9. Canetti, G.; Rist, N.; Grosset, J. Measurement of sensitivity of the tuberculous bacillus to antibacillary drugs by the method of proportions. Methodology, resistance criteria, results and interpretation. Rev. Tuberc. Pneumol. 1963, 27, 217–272.

10. Barrera, L.; Cooreman, E.; de Dieu Iragena, J.; Drobniewski, F.; Duda, P.; Havelkova, M.; Hoffner, S.; Kam, K.M.; Kim, S.J.; Labelle, S.; et al. Policy Guidance on Drug-Susceptibility Testing (DST) of Second-Line Antituberculosis Drugs; WHO Guidelines Approved by the Guidelines Review Committee; World Health Organization: Geneva, Switzerland, 2008.

11. Kam, K.M.; Sloutsky, A.; Yip, C.W.; Bulled, N.; Seung, K.J.; Zignol, M.; Espinal, M.; Kim, S.J. Determination of Critical Concentrations of Anti-Tuberculosis Drug Resistance from Whole-Genome Sequences. Clin. Diag. Microbiol. 2010, 14, 280–288.

12. Phelan, J.E.; O’Sullivan, D.M.; Machado, D.; Ramos, J.; Oppong, Y.E.A.; Campino, S.; O’Grady, J.; McNerney, R.; Hibberd, M.L.; Viveiros, M.; et al. Integrating Informatics Tools and Portable Sequencing Technology for Rapid Detection of Resistance to Anti-Tuberculous Drugs. Genome Med. 2019, 11, 41. [CrossRef] [PubMed]

13. Coll, F.; McNerney, R.; Preston, M.D.; Guerra-Assunção, J.A.; Warry, A.; Hill-Cawthorne, G.; Mallard, K.; Nair, M.; Miranda, A.; Alves, A.; et al. Rapid Determination of Anti-Tuberculosis Drug Resistance from Whole-Genome Sequences. Genome Med. 2015, 7, 51. [CrossRef] [PubMed]

14. Feuerriegel, S.; Schleusener, V.; Beckert, P.; Kohl, T.A.; Miotto, P.; Cirillo, D.M.; Cabibbe, A.M.; Niemann, S.; Fellenberg, K. PhyResSE: A Web Tool Delineating Mycobacterium Tuberculosis Antibiotic Resistance and Lineage from Whole-Genome Sequencing Data. J. Clin. Microbiol. 2015, 53, 1908–1914. [CrossRef] [PubMed]

15. Manson, A.L.; Cohen, K.A.; Abeel, T.; Desjardins, C.A.; Armstrong, D.T.; Barry, C.E.; Brand, J.; Chapman, S.B.; Cho, S.-N.; et al. TBResist Global Genome Consortium. Genomic Analysis of Globally Diverse Mycobacterium Tuberculosis Strains Provides Insights into the Emergence and Spread of Multidrug Resistance. Nat. Genet. 2017, 49, 395–402. [CrossRef]

16. Vilchêze, C.; Jacobs, W.R. Resistance to Isoniazid and Ethionamide in Mycobacterium Tuberculosis: Genes, Mutations, and Causalties. Microbiol. Spectr. 2014, 2, 2–4. [CrossRef]

17. Leung, K.L.; Yip, C.W.; Yeung, Y.L.; Wong, K.L.; Chan, W.Y.; Chan, M.Y.; Kam, K.M. Usefulness of Resistant Gene Markers for Predicting Treatment Outcome on Second-Line Anti-Tuberculosis Drugs. J. Appl. Microbiol. 2010, 109, 2087–2094. [CrossRef]

18. Coll, F.; Phelan, J.; Hill-Cawthorne, G.A.; Nair, M.B.; Mallard, K.; Ali, S.; Abdallah, A.M.; Alghamdi, S.; Alsomali, M.; Ahmed, A.O.; et al. Genome-Wide Analysis of Multi- and Extensively Drug-Resistant Mycobacterium Tuberculosis. Clin. Microbiol. Infect. 2019, 25, 1041.e1–1041.e7. [CrossRef]

19. Islam, M.M.; Tan, Y.; Hameed, H.M.A.; Liu, Z.; Chhotaray, C.; Liu, Y.; Lu, Z.; Cai, X.; Tang, Y.; Gao, Y.; et al. Detection of Novel Mutations Associated with Independent Resistance and Cross-Resistance to Isoniazid and Prothionamide in Mycobacterium Tuberculosis Clinical Isolates. Clin. Microbiol. Infect. 2019, 25, 1041.e1–1041.e7. [CrossRef]

20. WHO. Catalogue of Mutations in Mycobacterium Tuberculosis Complex and Their Association with Drug Resistance; WHO: Geneva, Switzerland, 2021.

21. Coll, F.; McNerney, R.; Guerra-Assunção, J.A.; Glynn, J.R.; Perdigão, J.; Viveiros, M.; Portugal, I.; Pain, A.; Martin, N.; Clark, T.G. A Robust SNP Barcode for Typing Mycobacterium Tuberculosis Complex Strains. Nat. Commun. 2014, 5, 4812. [CrossRef]

22. Engohang-Ndong, J.; Baillat, D.; Aumercier, M.; Bellefontaine, F.; Besra, G.S.; Locht, C.; Baulard, A.R. Ehr, a Repressor of the TetR/CamR Family Implicated in Ethionamide Resistance in Mycobacteria, Octamerizes Cooperatively on Its Operator. Mol. Microbiol. 2004, 51, 175–188. [CrossRef]

23. Dover, L.G.; Corsino, P.E.; Daniels, I.R.; Cocklin, S.L.; Tatituri, V.; Besra, G.S.; Fütterer, K. Crystal Structure of the TetR/CamR Family Repressor Mycobacterium Tuberculosis EthR Implicated in Ethionamide Resistance. J. Mol. Biol. 2004, 340, 1095–1105. [CrossRef]
24. Blondiaux, N.; Moune, M.; Desroses, M.; Frita, R.; Mathys, V.; Soetaert, K.; Kiass, M.; Delorme, V.; Djaout, K.; et al. Reversion of Antibiotic Resistance In Mycobacterium Tuberculosis by Spiroisoxazoline SMART-420. *Science* 2017, 355, 1206–1211. [CrossRef]

25. Flipo, M.; Frita, R.; Bourotte, M.; Martinez-Martinez, M.S.; Boesche, M.; Boyle, G.W.; Derimanov, G.; Drewes, G.; Gamallo, P.; Ghidelli-Disse, S.; et al. The Small-Molecule SMART751 Reverses Mycobacterium Tuberculosis Resistance to Ethionamide in Acute and Chronic Mouse Models of Tuberculosis. *Sci. Transl. Med.* 2022, 14, eaaz6280. [CrossRef]

26. Centre National de Rénovation des Mycobactéries et de la Résistance des Mycobactéries aux Antituberculeux Rapport Annuel D’activité. Available online: http://cnrmyctb.free.fr/IMG/pdf/rapport-CNR-MyRMA-2019b_web.pdf (accessed on 16 July 2021).

27. Coll, F.; Preston, M.; Guerra-Assunção, J.A.; Hill-Cawthorn, G.; Harris, D.; Perdigão, J.; Viveiros, M.; Portugal, I.; Drobniewski, F.; Gagneux, S.; et al. PolyTB: A Genomic Variation Map for Mycobacterium Tuberculosis. *Tuberculosis* 2014, 94, 346–354. [CrossRef]

28. Boonaiam, S.; Chaiprasert, A.; Prammananan, T.; Leechawengwongs, M. Genotypic Analysis of Genes Associated with Isoniazid and Ethionamide Resistance in MDR-TB Isolates from Thailand. *Clin. Microbiol. Infect.* 2010, 16, 396–399. [CrossRef]

29. Lee, H.; Cho, S.N.; Bang, H.E.; Lee, J.H.; Bai, G.H.; Kim, S.J.; Kim, J.D. Exclusive Mutations Related to Isoniazid and Ethionamide Resistance among Mycobacterium Tuberculosis Isolates from Korea. *Int. J. Tuberc. Lung Dis.* 2000, 4, 441–447.

30. Malinga, L.; Brand, J.; Jansen van Rensburg, C.; Cassell, G.; van der Walt, M. Investigation of Isoniazid and Ethionamide Cross-Resistance by Whole Genome Sequencing and Association with Poor Treatment Outcomes of Multidrug-Resistant Tuberculosis Patients in South Africa. *Int. J. Mycobacteriol.* 2016, 5 (Suppl. S1), S36–S37. [CrossRef]

31. Morlock, G.P.; Metchock, B.; Sikes, D.; Crawford, J.T.; Cooksey, R.C. EthA, InhA, and KatG Loci of Ethionamide-Resistant Clinical Mycobacterium Tuberculosis Isolates. *Antimicrob. Agents Chemother.* 2003, 47, 3799–3805. [CrossRef]

32. Rueda, J.; Realpe, T.; Mejia, G.I.; Zapata, E.; Rozo, J.C.; Ferro, B.E.; Robledo, J. Genotypic Analysis of Genes Associated with Independent Resistance and Cross-Resistance to Isoniazid and Ethionamide in Mycobacterium Tuberculosis Clinical Isolates. *Antimicrob. Agents Chemother.* 2015, 59, 7805–7810. [CrossRef]

33. Lefford, M.J.; Mitchison, D.A. Comparison of Methods for Testing the Sensitivity of Mycobacterium Tuberculosis to Ethionamide. *Tubercle* 1966, 45, 250–261. [CrossRef]

34. Schön, T.; Juréen, P.; Chryssanthou, E.; Giske, C.G.; Sturegård, E.; Kahlmeter, G.; Hoffner, S.; Angeby, K.A. Wild-Type Distributions of Seven Oral Second-Line Drugs against Mycobacterium Tuberculosis. *Int. J. Tuberc. Lung Dis.* 2011, 15, 502–509. [CrossRef]

35. Lakshmi, R.; Ramachandran, R.; Syam Sundar, A.; Rehman, F.; Radhika, G.; Kumar, V. Optimization of the Conventional Minimum Inhibitory Concentration Method for Drug Susceptibility Testing of Ethionamide. *Int. J. Mycobacteriol.* 2013, 2, 29–33. [CrossRef]

36. Kaniga, K.; Cirillo, D.M.; Hoffner, S.; Ismail, N.A.; Kaur, D.; Lounis, N.; Metchock, B.; Pfyffer, G.E.; Venter, A. A Multilaboratory, Multicountry Study To Determine MIC Quality Control Ranges for Phenotypic Drug Susceptibility Testing of Selected First-Line Antituberculosis Drugs, Second-Line Injectables, Fluoroquinolones, Clazofamidine, and Linezolid. *J. Clin. Microbiol.* 2016, 54, 2963–2968. [CrossRef] [PubMed]

37. Grant, S.S.; Wellington, S.; Kawate, T.; Desjardins, C.A.; Silvis, M.R.; Vivag, C.; Thompson, M.; Gordon, K.; Kazeyanskaia, E.; Nietupski, R.; et al. Baeyer-Villiger Monoxygenases EthA and MymA Are Required for Activation of Replicating and Non-Replicating Mycobacterium Tuberculosis Inhibitors. *Cell Chem. Biol.* 2016, 23, 666–677. [CrossRef] [PubMed]

38. Hicks, N.D.; Carey, A.F.; Yang, J.; Zhao, Y.; Fortune, S.M. Bacterial Genome-Wide Association Identifies Novel Factors That Contribute to Ethionamide and Prothionamide Susceptibility in Mycobacterium Tuberculosis. *MBio* 2019, 10, e00616-19. [CrossRef] [PubMed]

39. Grau, T.; Selchow, P.; Tigges, M.; Burri, R.; Gitzinger, M.; Böttger, E.C.; Fussenegger, M.; Sander, P. Phenylyethyl Butyrate Enhances the Potency of Second-Line Drugs against Clinical Isolates of Mycobacterium Tuberculosis. *Antimicrob. Agents Chemother.* 2012, 56, 1142–1145. [CrossRef]