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Genome sequencing of *Mycobacterium tuberculosis* clinical isolates revealed isoniazid resistance mechanisms undetected by conventional molecular methods

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ABSTRACT

A combination of targeted molecular methods and phenotypic drug-susceptibility testing is the most widely used approach to detect drug resistance in *Mycobacterium tuberculosis* isolates. We report the delay in the introduction of an efficient anti-tuberculous drug regimen because of a *M. tuberculosis* strain displaying a high level of resistance to isoniazid, in the absence of the common mutations associated with isoniazid-resistance, including *katG* mutations and *inhA* promoter mutations. Whole-genome sequencing (WGS) identified a large loss-of-function insertion (>1000 pb) at the end of *katG* in the isolate together with a −57C>T *ahpC* mutation, a resistance mechanism that would have remained undetected by a conventional molecular targeted approach. A retrospective search using publicly available WGS data of more than 1200 isoniazid-resistant isolates and a similar sized control dataset of isoniazid-susceptible isolates revealed that most (22/31) isoniazid-resistant, KatG loss-of-function mutants had an associated rare *ahpC* promoter mutation. In contrast, only 7 of 1411 isoniazid-susceptible strains carried a rare *ahpC* promoter mutation, including shared mutations with the 31 isoniazid-resistant KatG loss-of-function mutants. These results indicate that rare *ahpC* promoter mutations could be used as a proxy for investigating simultaneous KatG loss-of-function or missense mutations. In addition, WGS in routine diagnosis would improve drug susceptibility testing in *M. tuberculosis* clinical isolates and is an efficient tool for detecting resistance mechanisms undetected by conventional molecular methods.

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1. Introduction

Despite global efforts to eradicate tuberculosis, the disease continues to infect and kill millions of people in the world every year with 10.4 million new cases in 2017 and about 1.7 million deaths [1]. Successful treatment requires combinations of several drugs in addition to adequate prescription, drug quality, compliance, and good drug absorption. Introducing a targeted, effective therapy is important to avoid the selection of resistant bacteria and to shorten the long anti-tuberculous therapy that necessitates a combination of antibiotics with potentially significant side effects. Precise and reliable antibiotic susceptibility testing is paramount for efficient anti-tuberculosis treatment to prevent treatment failure, to minimize drug side effects and to prevent the emergence of drug-resistant tuberculosis [2].

Many efforts have been made towards accelerating diagnosis of tuberculosis, particularly molecular polymerase chain reaction (PCR)-based methods to circumvent the long turn-around time of *Mycobacterium tuberculosis* culture [3–7]. Antibiotic susceptibility testing in mycobacteria currently includes a combination of genotypic and phenotypic tests to guide clinicians in their therapeutic choices. In the last decade, whole-genome sequencing (WGS) emerged as a reliable approach to predict drug resistance in *M. tuberculosis*. WGS is increasingly used to predict antibiotic resistance of *M. tuberculosis* with excellent sensitivity and specificity, with the potential to complete or replace conventional molecular methods

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and phenotypic drug-susceptibility testing (DST) used in clinics for first-line antibiotics [8,9].

Isoniazid (INH) is a pro-drug activated in *M. tuberculosis* cytoplasm by the enzyme KatG, a catalase-peroxidase encoded by the *katG* gene [10]. Once activated, the drug inhibits the synthesis of mycolic acids forming the cell wall by inactivation of the enzyme InhA. The main mechanisms of resistance to isoniazid include (i) the alteration of KatG function, preventing the activation of the pro-drug, or (ii) the increased expression of InhA. The well characterized KatG Ser315 and *fabG1* −15C mutations were observed in 64% and 19% of isolates, respectively, with an isoniazid-resistant (INH-R) phenotype according to a meta-analysis of more than 11 000 isolates [11]. The catalase-peroxidase also plays an important role in *M. tuberculosis* fitness by protecting the bacterium from host-mediated oxidative stress. However, KatG Ser315Thr mutants show no impaired virulence during infections [12,13] and keep its full catalase-peroxidase activity [14–16].

Mutations in *katG* leading to dramatic downregulation of gene expression or impairment of the encoded protein activity have also been associated with isoniazid resistance [17–21]. Brossier et al. [16] investigated the different catalytic activities of 16 *katG* mutants. They confirmed that the Ser315Thr mutant showed a strong reduction of isoniazid activation but milder reduction in the peroxidase activity, as previously demonstrated [15]. However, a *katG* mutant bearing a distal stop mutation (Arg595*, leading to the truncation of 145 amino-acids) showed both reduced isoniazid activation and peroxidase activity. One of the proposed mechanisms for rescuing peroxidase activity for KatG loss-of-function mutants is the overexpression of AhpC through mutations in the promoter region of *ahpC* [22]. Although *ahpC* expression is repressed in virulent strains of *M. tuberculosis* and *M. bovis* [23], overexpression in INH-R *M. tuberculosis* isolates was reported a long time ago [24]. As isoniazid-susceptible (INH-S) strains overexpressing *ahpC* do not acquire isoniazid resistance [25], using *ahpC* promoter mutations for phenotypic prediction of isoniazid resistance is widely debated [14]. Moreover, many *ahpC* promoter mutations are also found in isoniazid-susceptible isolates [26]. Thus, interpreting isoniazid resistance in the presence of *ahpC* promoter mutation but in the absence of associated *katG* mutation is challenging [27].

We report the delay in the introduction of an optimal antituberculous drug regimen for a patient harboring a *M. tuberculosis* strain exhibiting a high level of resistance to isoniazid that was undetected by a conventional molecular targeted approach. After characterizing the molecular mechanism of resistance using high-throughput sequencing, its frequency in INH-R isolates was determined using publicly available genotype-phenotype datasets.

2. Material & methods

2.1. Tuberculosis microbiology diagnostics and drug susceptibility test

Sputum samples collected from the patient were analysed by microscopy, molecular diagnostics and culture as previously described [7,28]. Smear microscopy was performed from liquefied samples by the auramine fluorochrome method. Each sample was also tested by the Xpert MTB/RIF Ultra (Cepheid, California) [7,28]. Mycobacterial culture was performed on liquefied samples, decontaminated and inoculated in a mycobacterial growth indicator tube (MGIT).

Phenotypic drug susceptibility testing (DST) for first-line drugs - rifampin (RIF), isoniazid (INH), streptomycin (STR), ethambutol (EMB) and pyrazinamide (PZA) - was also performed using the MGIT method. Second-line DST was carried out at the Institute of Medical Microbiology, University of Zurich, Switzerland, as previously described [2].

Genotypic DST was performed directly from the clinical samples and then from the MGIT-positive culture medium by PCR amplification and sequencing. The clinical samples were processed by MagNAPure (Roche Life Science, Switzerland) for DNA extraction. The chromosomal DNA from bacterial strains was extracted from 1 mL of heat-inactivated positive MGIT with the QIAmp mini Kit (Qiagen, Germany). Hotspot regions of resistance-inducing mutations in *rpoB, fabG1* and *katG* were routinely amplified and Sanger-sequenced [29] on DNA from both clinical samples and positive MGIT. The *ahpC* promoter region was amplified and sequenced on DNA from the positive MGIT only. Specific primers targeting the 3'-end of *katG* were designed to confirm the high-throughput sequencing results (Forward primer: GCCCAGCGGTAAGCGCTT, Reverse primer: TGGCTTCCGAAACTACCTCG).

2.2. Whole-genome sequencing data

DNA libraries were prepared using the Nextera XT DNA library preparation kit (Illumina, San Diego, California) on the DNA extracted from the positive MGIT. Quality was checked using a Fragment Analyser (Advanced Analytical Technologies, Inc, AATI, US) before sequencing on a MiSeq (Illumina, San Diego, California). Raw sequencing data were submitted to Sequence Read Archive (SRA, NCBI) under the project accession PRJNA634983.

Three public datasets referencing phenotypic resistance and raw genomic data of *M. tuberculosis* isolates were used to study the association between *katG* loss-of-function and *ahpC* compensatory mutations. Raw data were obtained from the Sequence Read Archive (SRA, NCBI) for isoniazid resistant and susceptible isolates phenotyped in TB Portals https://tbportals.niaid.nih.gov/, Walker et al., 2015 [8] and by the CRyPTIC consortium [9]. To extract data from TB Portals, the HTML page of every entry was automatically downloaded and parsed to extract the phenotypic and genetic data for each isolate. Supplementary materials from [8] and [9] were used to access the data associated with the papers.

2.3. Bioinformatic analyses

Paired-end sequence reads from the isolate and SRA were mapped on the H37Rv reference genome (accession NC_000962.3) and variants called using GATK's HaplotypeCaller [30] and freebayes [31]. Variants were annotated using SnpEff [32] against NC_000962.3. SPAdes [33] was used to assemble the genome of the local isoniazid-resistant isolate. Visualization of the assembly graph was performed with Bandage [34]. Visualization of the read mappings was performed with the Integrative Genomics Viewer [35]. We defined as a rare *ahpC* promoter mutation any mutation in the *ahpC-oxyR* intergenic region according to the annotation of NC_000962.3, excluding the well-known −88G>A polymorphism [26], which was found in more than 25% of the isoniazid-susceptible isolates investigated in this study.

3. Results

3.1. Clinical case: isoniazid-resistant strain undetected by conventional targeted molecular methods

An adult patient who self-reported 6 months of treatment for tuberculosis 10 years ago presented with a chronic cough and clinical symptoms indicating a possible reinfection or a relapse. The Xpert MTB/RIF Ultra performed the same day (day 0) detected DNA of *M. tuberculosis* complex at a medium quantity with no mutation in the *rpoB* gene (Figure 1). Smear microscopy showed a significant number of acid fast bacilli (1-10 AFB/microscopic field). Sanger sequencing of *rpoB, fabG1* and *katG* directly on the clinical sample did not reveal mutations known to be associated with rifampicin

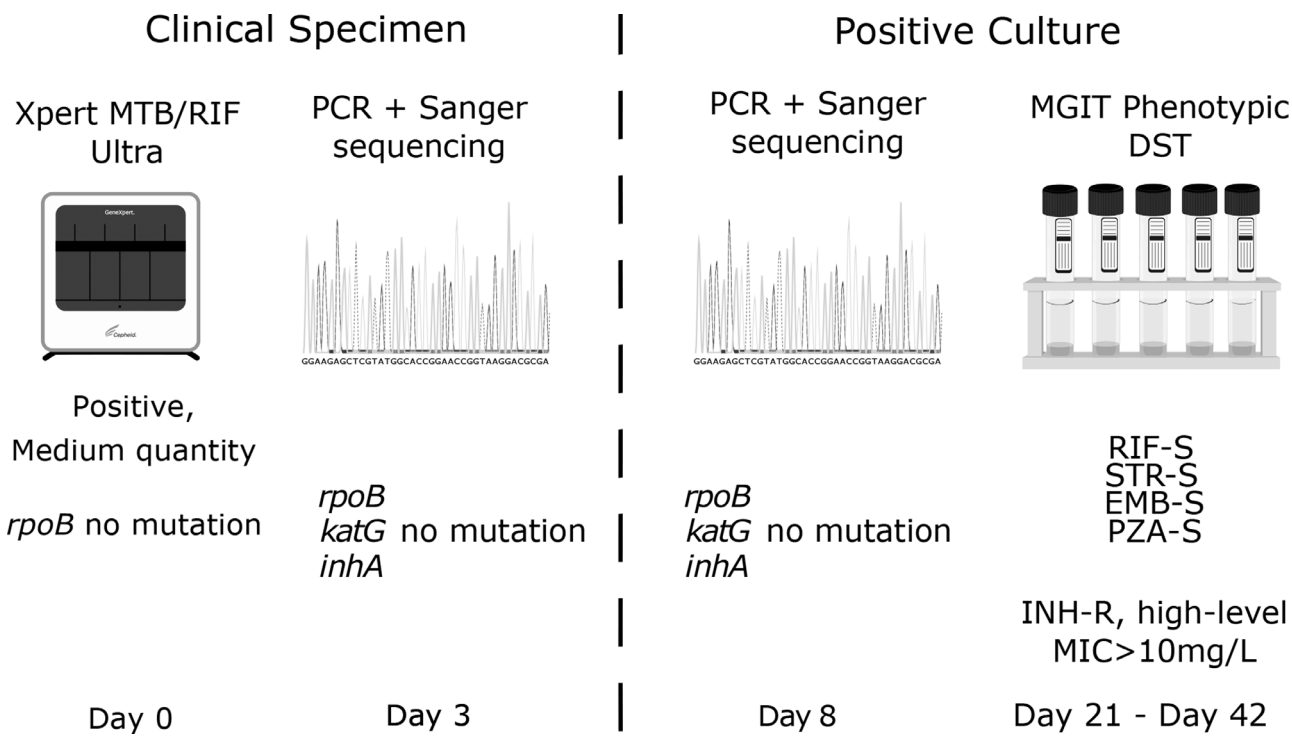

Figure 1. Schematic timeline of the clinical case investigated. No markers of resistance were identified before the phenotypic drug susceptibility test after mycobacterial growth.

or isoniazid resistance (Supplementary Figure 1). The patient was prescribed standard quadri-therapy of RIF, INH, EMB and PZA for susceptible tuberculosis. After 8 days of incubation, the mycobacterial culture turned positive for *M. tuberculosis* complex. Sequencing of *rpoB, fabG1* and *katG* on the DNA extracted from the liquid culture was identical to the sequencing on the clinical sample. After 21 days, the phenotypic DST revealed that the strain was susceptible to RIF, which was consistent with the genetic prediction. The strain was also susceptible to STR, PZA and EMB. However, the phenotypic DST showed a high level of resistance to INH (minimum inhibitory concentration [MIC] >10 mg/L). Repeated phenotypic DSTs at days 32 and 42 showed identical results. The patient was eventually treated with RIF, PZA, EMB, moxifloxacin (MOX) and amikacin for 2 months followed by treatment with RIF, PZA, EMB and MOX.

3.2. Investigation of the molecular basis of isoniazid resistance

Sanger sequencing on the DNA extracted from the positive MGIT identified a −57C>T mutation in the *ahpC* promoter region previously associated with isoniazid resistance [8]. The *ahpC* upstream mutation was confirmed by WGS, which also showed a large insertion at the 3'-end of the *katG* gene. The insertion was first identified during investigation of the read mappings against the reference H37Rv genome with IGV (Supplementary Figure 2), which showed an anomaly in the estimated fragment length of the read pairs at the end of *katG*. Visualizing the *de novo* genome assembly with Bandage showed that the two contigs harboring the two halves of *katG* were linked by a contig bearing the IS6110 sequence and two small contigs (Figure 2A). Sanger sequencing of amplicons obtained by PCR targeting the predicted insertion site confirmed the insertion of the IS6110 mobile element (Figure 2B and Supplementary Figure 3). Sequence analysis showed that the mobile element integration on *katG* caused the insertion of 29 codons together with a stop codon after codon number 602 (p.Asn602_Pro603ins29Ter). We hypothesized that the early stop codon led to a loss-of-function of KatG that was compensated by AhpC expression through the −57C>T promoter mutation. An isoniazid-resistant isolate analysed for an external quality control that had a −51G>A *ahpC* mutation found by Sanger sequencing was then reassessed. WGS also revealed a nonsense mutation at the beginning of *katG* (p.Trp90*) in this isolate.

3.3. Characterization of similar isoniazid resistance mechanisms using publicly available datasets

More than 1200 isoniazid-resistant strains for which WGS data were available were genotyped to characterize the frequency of this resistance mechanism. Thirty one isolates carried a loss-of-function mutation in *katG* (Tables 1 and 2): 10 early stop codons, 6 small frameshift insertions or deletions, 3 start codon mutations, 7 large deletions encompassing the *katG-furA* locus, one large mobile element insertion at the beginning of *katG*, one mixed sample presenting both an early stop codon and a full deletion of the locus and one mixed sample presenting both a frameshift and an early stop codon. Of the 31 total isolates, 22 carried 15 different rare mutations in the *ahpC* promoter (Tables 1 and 2, Figure 3), three of which (−72C>T, −52C>T and −48G>A) were also found in isoniazid-susceptible isolates. The remaining 9 isolates carried a clear loss-of-function mutation in *katG*, including an early frameshift mutation and a complete deletion of the locus, with no associated *ahpC* promoter mutation, raising the possibility of alternative rescue mechanisms. Overall, *katG* mutations associated with loss-of-function were found in a very low proportion of isoniazid-resistant isolates.

However, every (59/59, Table 2) isoniazid-resistant isolate with rare *ahpC* promoter mutations presented either loss-of-function (Table 1), missense or in-frame insertion (Supplementary Table 2 and 3) *katG* mutations. Twenty-six isolates carried both rare *aphC* promoter mutation and missense mutations in *katG* affecting a codon other than Ser315 (Supplementary Table 3). Most of these mutations have already been described in the context of isoni-

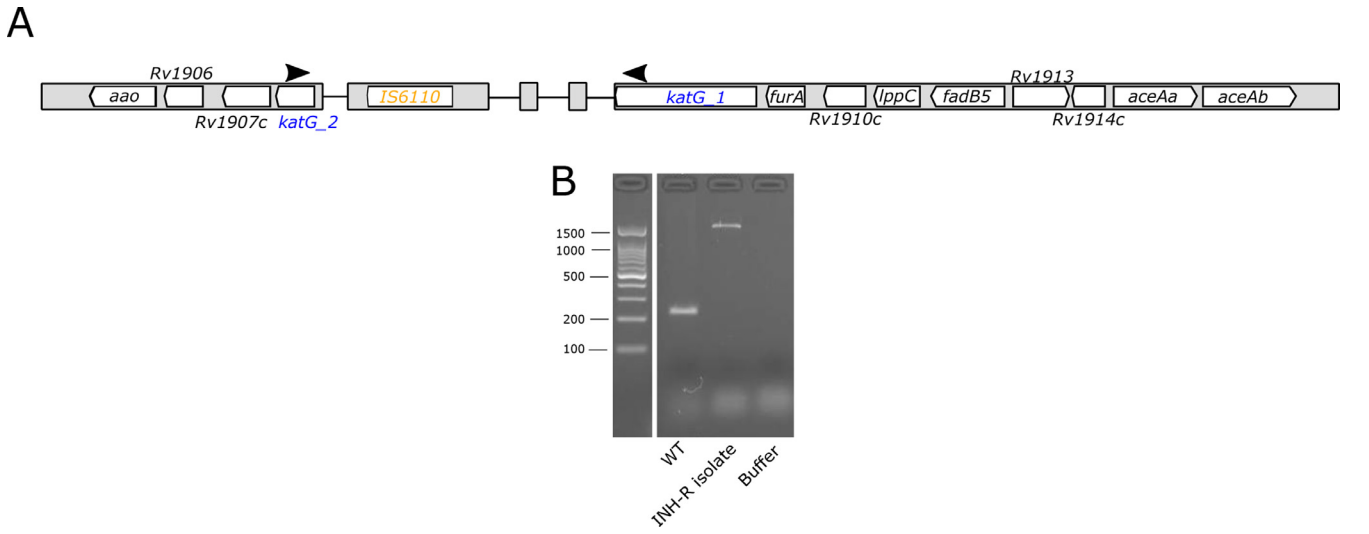

Figure 2. Summary of the molecular investigations. A: Schematic representation of the *katG* locus in our *de novo* genome assembly. The *katG* gene is split onto two different contigs linked by two small contigs (128 bp long each) and one bearing the IS6110 transposase coding sequence (1355 bp long) according to the Bandage visualization. Arrows represent the primers-targeted regions for PCR confirmation. Contigs bearing the two halves of *katG* are not shown entirely. B: Gel of the PCR products of the isoniazid-resistant isolate and controls, using the primers targeting the end of *katG*, showing a longer product in our isolate, the estimated size of which matched the length of the predicted insertion. Sanger sequencing confirmed the identity of the transposase.

Table 1
Occurrences of loss-of-function *katG* mutations and their associated rare *ahpC* promoter mutations. [1] Mutations seen in the INH-S dataset.

Sample	*katG* mutation type	*katG* mutation size	*katG* mutation annotation	*ahpC* mutation annotation
SAMEA104172498	Start codon lost	SNV	Val1Ala	−52C>T[1]
SAMEA104172498	Start codon lost	SNV	Val1Ala	No mutation
SAMEA1101554	Start codon lost	SNV	Val1Ala	No mutation
SAMEA2535041	Frameshift	SNV	Ile8fs	No mutation
SAMEA2534816	Stop	SNV	Tyr28*	−88_−87insAT
M74325 (this study)	Stop	SNV	Trp90*	−51G>A
SAMEA2534074	Frameshift	SNV	Gly124fs	−74G>A
SAMEA3392615	Stop	SNV	Trp149*	−54C>T
SAMEA2533767	Stop	SNV	Trp149*	−52C>A
SAMEA2533723	Frameshift	SNV	Glu342fs	−54C>T
SAMEA3392615	Stop	SNV	Trp351*	−90G>A
SAMEA2533962 (mixed sample)	Frameshift;Stop	SNV	Trp351fs;Arg595*	−48G>A
SAMN07236260	Stop	SNV	Gln352*	−51G>A
SAMEA2709836 (mixed sample)	Stop; Deletion	SNV; 40 kbp	Arg418*; Overlaps the full *katG-furA* locus	No mutation
SAMEA1101756	Stop	SNV	Trp438*	−76T>A and −76T>C
SAMEA2533599	Frameshift	SNV	Ile455fs	No mutation
SAMEA104172495	Stop	SNV	Trp477*	−81C>T
SAMEA2534002	Stop	SNV	Trp505*	No mutation
SAMEA2297114	Frameshift	SNV	Asn602fs	No mutation
SAMEA1018848	Frameshift	SNV	Leu634fs	No mutation
SAMEA3392615	Stop	SNV	Trp668*	−81C>T
SAMEA3252929	Stop	SNV	Ser671*	−48G>A[1]
SAMEA3359352	Insertion	around 1kbp	Mobile element insertion at beginning of *katG*	−52C>T[1]
180115 (this study)	Insertion	around 1kbp	Mobile element insertion at end of *katG*	−57C>T
SAMEA3303367 (mixed sample)	Deletion	39 kbp	Overlaps the full *katG-furA* locus	−72C>T[1];−52C>T[1];−48G>A[1]
SAMEA1102329	Deletion	23 kbp	Overlaps the full *katG-furA* locus	−76T>G
SAMEA1101781	Deletion	16 kbp	Overlaps the full *katG-furA* locus	No mutation
SAMEA1101769	Deletion	5 kbp	Overlaps the full katG-furA locus	−76T>A
SAMEA2533545	Deletion	400 bp	Across the end of *katG*	−77T>G
SAMEA2534034	Deletion	3.8 kbp	Includes promoter region of *katG*	−57C>T
SAMEA2534152	Deletion	24.5 kbp	Starts at the end of *katG*	−72C>T[1]

Table 2
Overview of the genotyping of publically available INH-R and INH-S isolates.

	INH-R isolates	INH-S isolates
Total	1233	1411
KatG loss-of-function mutants	31	NA
Rare ahpC promoter mutation	59	7
Rare ahpC promoter + loss-of-function katG mutations	22	0
Rare ahpC promoter + Ser315 katG mutations	11	0
Rare ahpC promoter + other missense katG mutations	26	1
Rare ahpC promoter + no katG mutation	0	6

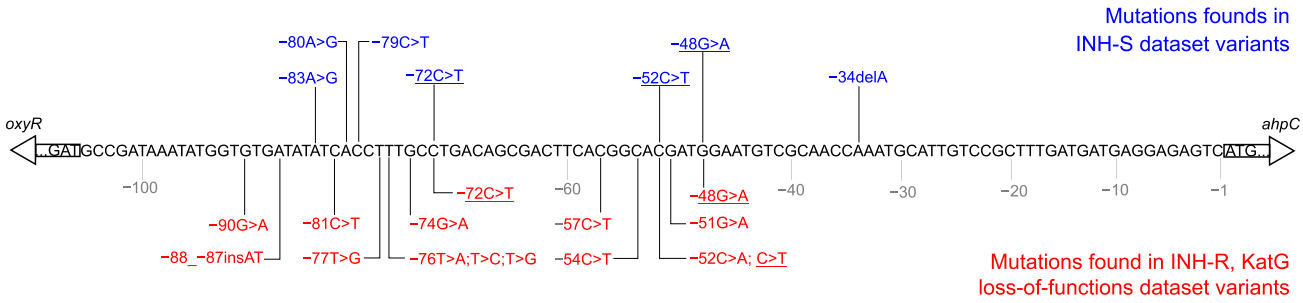

Figure 3. Mutations found in this study in the *ahpC* promoter, based on the sequence and annotation of NC_000962.3. Mutations above the DNA sequence were found in the isoniazid-susceptible dataset whereas mutations below were found in isoniazid-resistant isolates carrying loss-of-function *katG* mutations. The frequent polymorphism −88 G>A is not shown.

azid resistance [14]. In contrast, in the control dataset of 1411 isoniazid-susceptible *M. tuberculosis* genotypes, only seven isolates carried a rare *ahpC* promoter mutation (Table 2). As well as the three aforementioned mutations found in our isoniazid-resistant isolates with *katG* loss-of-function mutations, four other rare *ahpC* promoter mutations were identified (Figure 3 and Supplementary Table 1). None of these seven isoniazid-susceptible strains carried *katG* loss-of-function mutations but one showed a missense mutation of unknown significance (Gly124Ala).

4. Discussion

Herein is described a case of high-level resistance to isoniazid revealed by culture-based phenotypic DST that was undetected by a conventional molecular targeted approach. Using PCR and Sanger sequencing, no mutations commonly associated with isoniazid resistance in *fabG1* or *katG* were found in the sequences amplified from the clinical sample or in the sequences amplified from the strains recovered after culture. However, PCR and Sanger sequencing identified a −57C>T *ahpC* promoter mutation known to rescue *katG* function [36], and this was confirmed by WGS. In addition, WGS showed a large loss-of-function insertion (>1 kpb, IS6110) in *katG,* subsequently confirmed by Sanger sequencing. Altogether these data indicate isoniazid resistance mediated by a *katG* loss-of-function mutation, compensated by the activation of AhpC through a mutation in the promoter region of its gene [36].

Rapid and accurate identification of resistance patterns of *M. tuberculosis* strains is essential for the treatment and management of tuberculosis-infected patients. Inaccurate drug susceptibility tests leading to undertreatment of drug-resistant *M. tuberculosis* strains have a significant impact on mortality [37]. Culture-based phenotypic drug susceptibility testing in *M. tuberculosis* has always been challenging because of the slow growth rate of its fastidious bacteria and to the heterogeneity of its population. However, it still serves as reference to determine *M. tuberculosis* drug susceptibility, with an estimated delay of weeks and even months in the case of MDR- and XDR-TB to obtain a comprehensive drug susceptibility test.

Molecular approaches that enable drug resistance prediction to be achieved directly on clinical specimen or on positive culture have been developed to circumvent *M. tuberculosis* slow growth. These molecular assays improved the detection of MDR-TB and patient management. Although simple and rapid, these tests have several drawbacks. Commercial molecular tests, such as Xpert MTB/RIF, Xpert MTB/RIF Ultra or line probe assay, indicate the resistance for a limited number of selected drugs and detect known mutations [4,5,28]. For instance, the GeneXpert MTB/RIF Ultra detects rifampicin resistance only. Furthermore, the line probe assays detect the most frequent *katG* mutations that lead to isoniazid resistance patterns but sometimes fail to detect isoniazid mono-resistance, which is common in MTB strains [38].

WGS accesses the entire genome of *M. tuberculosis*, providing information on single nucleotide variants, small insertion/deletions and large structural variation in both coding and intergenic regions. This technology is now available in most modern laboratories with a time to results of 48 to 72 h depending on the laboratory facilities. Consequently, WGS can rapidly predict drug resistance with high accuracy [8,9] and detect yet undescribed genetic variation that could be associated with drug resistance. Recent advances in high-throughput sequencing make this technology cost-effective, and fast enough for application in routine diagnostic laboratories. This technology is often used only for specific situations or as a routine tool only in a limited number of settings [2].

WGS detects structural variations, such as large insertions or deletions, within or in the vicinity of the *katG* gene. The occurrence of such resistance mechanisms was investigated by analysing *M. tuberculosis* genomes available in public databases. This identified 20 additional isoniazid-resistant isolates presenting low frequency *ahpC* promoter mutation together with *katG* loss-of-function mutations, including structural variants.

The unexpected molecular basis of the resistance to isoniazid in the clinical isolate in this study indicated that such a mechanism might remain undetected by a targeted molecular approach, such as targeted Sanger sequencing or molecular probes-based assay, which might lead to undertreatment. WGS is now established as a reliable method for predicting susceptibility and resistance at least for first-line antibiotics. The current study indicates that some resistance mechanisms may remain uninvestigated even using WGS. This includes premature stop codons, frameshifts or large insertions within the *katG* gene but also structural variation in the vicinity of *katG*. Using genome assembly approaches, coverage analysis or discrepancies in estimated fragment length between paired reads, these structural variations in the vicinity of *katG* can be detected after high-throughput sequencing. When using this technology to predict isoniazid resistance, nonsense or frameshift mutations in the *katG* gene should be identified because they are likely leading to high-level resistance. Such variation is rare compared with KatG Ser315Thr or *fabG1* −15C>T mutations but can explain unresolved cases of high-level isoniazid resistance. Moreover, rare variants in the *aphC* promoter region associated with *katG* loss-of-function have been identified. Most of these variants were not present in the susceptible population analysed, raising the possibility of a fitness cost of these variants when *katG* is functioning normally.

Some mutations in the *ahpC* promoter, such as the −88G>A polymorphism, appeared not to be linked to isoniazid resistance. Such mutations might not be linked to any fitness advantage and should not be used for predicting resistance to isoniazid. Other *ahpC* promoter mutations are found in either isoniazid-susceptible

or -resistant isolates. Variants in this region present in both KatG loss-of-function mutants and susceptible isolates should also be interpreted with caution. Such variants could compensate for decreased KatG activity and subsequent acquisition of isoniazid resistance in isolates carrying them could be more likely. Future studies based on transcriptomic or phenotypic assays may address this hypothesis.

Recently, Kandler et al. [39] validated rare, missense *katG* mutations identified in isoniazid-resistant isolates. They also identified 19 KatG loss-of-function mutants (stops, frameshifts or deletions in the *katG-furA* locus), 10 of which showed single nucleotide variants in the *ahpC* promoter region. One of the *ahpC* mutations they identified was not found in the present study (−84_−83insTC); however, it is located in one of the two hotspots that include all other single nucleotide variants identified so far (Figure 3). Moreover, they found an additional case of apparent multiplication of the *ahpC* locus in the genome of one isolate. None of the 9 isoniazid-resistant, KatG loss-of-function mutants lacking an *ahpC* promoter mutation identified in this study showed this multiplication. Similar to the current analysis, they found several loss-of-function KatG mutant isolates without compensatory *ahpC* promoter mutations (8/19).

In this study, every isoniazid-resistant isolate carrying low frequency promoter *ahpC* mutations also carried loss-of-function or missense mutations in *katG* (59/59). One isolate, reported as isoniazid-susceptible displaying a rare *ahpC* promoter mutation carried a missense *katG* mutation. None of the isoniazid-susceptible isolate with a rare *ahpC* mutation carried a loss-of-function mutation. In their study investigating the different catalytic activities of *katG* mutants, Brossier et al. [16] also identified missense mutations showing reduction in both isoniazid activation and peroxidase activity. Mutations in the *ahpC* promoter rescuing peroxidase activity are predicted to be beneficial in isolates carrying these missense mutations. However, as peroxidase activity might be maintained in missense *katG* mutants leading to loss of isoniazid activation activity, the selective pressure for rescuing it might be lower than in the KatG loss-of-function mutants. Focusing on rare *ahpC* promoter mutation might therefore help in identifying *katG* missense mutants leading to complete loss-of-function of all the activities of the protein.

Rare *aphC* promoter mutations are overrepresented in the isoniazid-resistant dataset; therefore, screening these mutations might be useful prior to low frequency *katG* mutations screening and WGS. Consequently, this approach has been introduced in our setting for routine diagnostics. Furthermore, the results confirmed the usefulness of WGS to identify possible KatG loss-of-function mutations and to shorten the delays necessary to investigate discordant results between molecular tests and phenotypic DST. Further study of the co-occurrence of *ahpC* promoter and *katG* variants will help distinguish neutral *ahpC* variants from those promoting higher expression, which should be assessed in functional studies [39]. Future studies should also focus on such structural variations occurring in *katG* gene or in its vicinity in the absence of known compensatory mutations in the *ahpC* promoter. This may help identify other still unknown compensatory mechanisms that may be selected in the arms race between treatment of human infection and evolution of bacterial antimicrobial resistance.

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Declarations

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Competing Interests: No
Ethical Approval: Not required

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ijantimicag.2020.106068.

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