Introduction

Isoniazid (isonicotinic acid hydrazide) was first synthesized over a century ago (1912), but not until 1952 was its antituberculous activity exploited. Since then, isoniazid has become the most widely prescribed medication for the treatment of drug-susceptible TB, along with rifampicin and pyrazinamide. Moreover, isoniazid monotherapy has been promoted as a treatment for latent TB. The provision of so-called isoniazid preventive therapy is strongly endorsed for HIV-infected individuals who are unlikely to have developed active TB. Similar to other anti-TB agents, resistance to isoniazid emerged shortly after its introduction into clinical practice. The prevalence of isoniazid resistance has reached disturbingly high proportions over the last two decades. Globally, the combined prevalence of resistance to isoniazid, either alone or in combination with other drugs, has now exceeded 13%, which translates into every seventh TB case being resistant to isoniazid. This poses a serious threat to the management and control of TB across the world.

The mechanisms of resistance to isoniazid are complex and not fully elucidated. A pivotal role has been attributed to mutations at several loci of the Mycobacterium tuberculosis genome, including katG, inhA (with its promoter region mabA-inhA), kasA, ahpC (with the upstream regulatory region oxyR-ahpC), ndh, nat and mshA. Mutations in these genes may potentially be used as surrogate markers in molecular assays to detect isoniazid resistance in M. tuberculosis, even at the clinical sample level, thus offering a rapid and effective alternative to conventional drug susceptibility testing. A number of genotypic methods based on the detection of mutations known to mediate isoniazid resistance have recently been developed, including DNA sequencing, line probe assays, hybridization on DNA chips, single-strand conformation polymorphisms, pyrosequencing and real-time PCR. The

Mutation profiling for detection of isoniazid resistance in Mycobacterium tuberculosis clinical isolates

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Mutations conferring isoniazid resistance in M. tuberculosis

Accuracy and diagnostic utility of these methods rely heavily on the careful selection of mutations that would not only be strongly associated with the phenotypic resistance but also highly prevalent within the pathogen population. Therefore, the evaluation of the type and distribution frequency of isoniazid resistance-conferring mutations in different clinical and geographical settings is a prerequisite before genotype-based approaches for the detection of isoniazid resistance can be established on a large scale.

Previously, 50 strains of MDR M. tuberculosis were extensively characterized for the presence of mutations within nine genetic loci, i.e. seven structural genes (katG, inhA, ahpC, kasA, rhl, nat and mshA) and two regulatory regions (the mabA-inhA promoter and oxyR-ahpC intergenic region). In this study, 50 isoniazid-monoresistant and 50 isoniazid-susceptible M. tuberculosis clinical isolates were investigated using the same methodological approach (i.e. with the same set of loci and using the same analytical strategy) as for the MDR strains. For comparative reasons, the results obtained in this study and those previously published were collected and discussed. Therefore, the aim of this study was 2-fold: (i) to investigate the prevalence of isoniazid resistance-associated mutations in the two tubercle bacilli populations (MDR and isoniazid-monoresistant); and (ii) to compare mutation distribution patterns specifically between isoniazid-monoresistant and MDR strains.

Materials and methods

Strains and drug susceptibility testing

A total of 150 M. tuberculosis strains, deposited in the culture collection of the National Tuberculosis and Lung Diseases Research Institute in Warsaw, were used for the study. Included in that number were 50 MDR, 50 isoniazid-monoresistant and 50 pan-susceptible strains. The strains were recovered from different pulmonary TB patients (112 males and 38 females, age range of 14–93 years and median age of 51 years), diagnosed in TB dispensaries across Poland, and collected during the third national survey of drug-resistant TB (i.e. from 2004 to 2005). The study sample represented 100%, 61% and 1.7% of all bacteriologically confirmed MDR, isoniazid-monoresistant and drug-susceptible TB cases reported in Poland during the survey period. Primary isolation, cultivation and species identification were done by using standard mycobacteriological methods as described elsewhere. Susceptibility to isoniazid was measured with the 1% proportion method on Löwenstein–Jensen (LJ) medium at a critical drug concentration of 0.2 mg/L. The MIC of isoniazid was determined in LJ medium containing 2-fold incremental concentrations of the drug ranging from 0.05 to 60 mg/L. The MIC was defined as the lowest concentration of isoniazid that inhibited >99% of the bacterial growth as compared with a drug-free control.

Catalase activity

A standard semi-quantitative assay was used to evaluate catalase activity of all M. tuberculosis strains under study. The M. tuberculosis laboratory strain H37Rv was used as a positive control.

DNA extraction

Genomic DNA was extracted from M. tuberculosis cultures on LJ slants by the lysozyme/proteinase K cetyltrimethylammonium bromide method.

DNA sequencing and data analysis

For all strains, seven structural genes (katG, inhA, ahpC, kasA, rhl, nat and mshA) and two regulatory regions (the mabA-inhA promoter region and the oxyR-ahpC intergenic region) were sequenced in their entirety. The oligonucleotide primers and PCR amplification and sequencing conditions were as previously described. Sequencing, on both strands, was performed by Genomed (Poland) by using the BigDye version 3.1 Terminator Cycle Sequencing Kit (Applied Biosystems) in the ABI 3130xL Genetic Analyzer (Applied Biosystems). The sequence data were assembled and analysed by using ChromasPro (version 1.7.1) software (Technelysium) and consensus sequences were compared with the corresponding sequences of M. tuberculosis strain H37Rv (http://genolist.pasteur.fr/Tuberculist/) using the BLASTN algorithm (http://blast.ncbi.nlm.nih.gov/). To establish whether a given mutation had or had not been described previously, the mutated allele sequences were searched against the following interactive, publicly available databases: GenBank (National Center for Biotechnology Information; http://www.ncbi.nlm.nih.gov/), the TB Drug Resistance Mutation Database (http://www.tbdreamdb.com/), MuBII (https://umr5558-bibiserv.univ-lyon1.fr/mubii/mubi-select.cgi/) and the Broad Institute database (http://www.broad institute.org/annotation/genome/mtb_drug_resistance.1/DirectedSequencingHome.html/).

Nucleotide sequence accession numbers

The sequences with novel mutations found among isoniazid-monoresistant and drug-susceptible M. tuberculosis isolates were deposited in GenBank (http://www.ncbi.nlm.nih.gov/) under the following accession numbers: KP191532–KP191544 for the katG gene; KP40584 and KP642079 for the mabA-inhA promoter region; KP410582 and KP410583 for the inhA gene; KP743985 and KP743986 for the ahpC gene; KP642080 for the oxyR-ahpC regulatory region; KP743989, KP743990, KP230539 and KP230540 for the rhl gene; KP743987, KP743988 and KP230538 for the nat gene; and KP191545–KP191549 for the mshA gene.

Statistical analysis

The associations between variables (mutations, MICs of isoniazid and catalase activity) were assessed with the non-parametric χ² and Mann-Whitney U-test or (if the cell count was <5) Fisher's exact test. A P value of <0.05 was considered significant. For all analyses, the SPSS statistical software package (version 20.0; SPSS, USA) was used.

Results

A total of 66 distinct mutations were detected at all nine loci investigated, accounting for 109 (72.7%) of the strains tested (Table S1, available as Supplementary data at JAC Online). The number of strains with any mutation within the MDR, isoniazid-monoresistant and pan-susceptible strain subsets was 49 (98%), 37 (74%) and 23 (46%), respectively. Mutations in the katG gene predominated, with 29 different types distributed among 46 (92%) MDR, 31 (62%) isoniazid-monoresistant and 2 (4%) pan-susceptible strains. The most common katG mutation was a G944C transition, conferring a Ser315Thr amino acid change, found in 33 (66%) MDR and 21 (42%) isoniazid-monoresistant strains. In three strains, this mutation co-occurred with a C945T substitution, still resulting in the same Ser315Thr replacement. Of the remaining 27 katG mutation types, only 4 occurred in more than one isolate. These mutations were G1388T (Arg636Leu), G383A (Arg128Gln) and C701G (Ala234Gly), found in eight MDR strains in total, and A1197G (Glu399Glu), found in three isoniazid-monoresistant strains.

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Mutations in the mabA-inhA promoter region were detected in eight (16%) MDR strains (C–15T in seven strains and T–8C in one strain) and one susceptible strain (T–80G). Mutations in the inhA gene were of four types: mutations T289G (Ser94Ala) and G681A (Pro227Pro) were found in single MDR strains and mutation A170G (Lys57Arg) was found in one isoniazid-monoresistant strain, whereas mutation T581C (Ile194Thr) was found in one MDR strain and two isoniazid-monoresistant strains (in one it was the only mutation observed). One isoniazid-monoresistant strain had a double-mutated mabA allele, with mutations G143T (Gly48Val) and T224C (Val75Ala).

Of the four mutations within the oxyR-ahpC locus, three (C–54T, C–57T and TCA–82I–80ATC) were identified in single MDR strains, whereas mutation G–48A was found in two MDR strains and one isoniazid-monoresistant strain. Four different ahpC mutations were represented by single strains; mutations C487T (Arg163STOP) and C114G (Ile38Met) were found in isoniazid-monoresistant strains (with the former mutation being the only mutation observed in the strain), while mutations A342C (Ile114Ile) and G124C (Glu42Gln) were found in an MDR strain and a susceptible strain, respectively.

There were only two types of mutations at the kasA gene. The G961A (Ala321Thr) mutation was demonstrated in 1 MDR strain only and the other mutation (G805A, Gly269Ser) was detected in 11 (22%) MDR and 5 (10%) isoniazid-monoresistant strains (in 1 strain it was the only mutation observed).

Six different mutations in the ndh gene were found, four being non-silent mutations. Of these, three were represented by single susceptible (G458C, Ser153Thr), isoniazid-monoresistant (C148G, Leu50Val) and MDR (G989C, Ala300Pro) strains. One ndh type mutation (T53C, Val184Ala) was found in three isoniazid-monoresistant and two MDR strains.

Of the three non-silent mutations in the nat gene, two (G518A, Trp127STOP; and C308G, Ala103Gly) were demonstrated for MDR strains, whereas one (G619A, Gly207Arg) was found in three isoniazid-monoresistant and two MDR strains.

Seven mutation types were identified for the mshA gene, split among 18 (36%) pan-susceptible, 17 (34%) MDR and 12 (24%) isoniazid-monoresistant strains. The most frequently encountered alteration was A332G (Asn111Ser), which occurred in 11 (22%) MDR and 5 (10%) isoniazid-monoresistant strains (in 1 strain it was the only mutation observed).

Mutations in the katG gene were of four types: mutations T289G (Ser94Ala) and G681A (Pro227Pro) were found in single MDR strains and mutation A170G (Lys57Arg) was found in one isoniazid-resistant strain, whereas the identification of mutations that would exclusively occur in MDR strains, being absent in non-MDR isoniazid-resistant strains, could provide a marker for the MDR phenotype.

Globally, isoniazid resistance has most frequently been associated with mutations in the katG gene, with the Ser315Thr substitution predominating. According to a recent systematic review aimed at assessing the global prevalence of the most common mutations linked with isoniazid resistance, mutations in katG codon 315 occurred at the highest frequency of 64.2%. Mutations in katG codons other than 315 were rare, with their frequencies of detection <1%. Substantial geographical variation exists in the incidence of katG 315 mutations. Among isoniazid-resistant tubercle bacilli, these mutations were overwhelmingly represented in South Africa (97.5%), Russia (93.6%) and Lithuania (85.7%), highly prevalent in Australia (65.4%), China (64.4%), Brazil (61.9%) and the Netherlands (53%), but much less common in Japan (41%), Italy (37.8%) and the USA (31%). An observation, which has repeatedly been reported in the literature, is that the frequency of katG 315 mutations is considerably higher in MDR than isoniazid-monoresistant strains. The proportions of katG 315 mutations in those two strain populations have been calculated at 88.2% versus 66.6%, 27 86.4% versus 61.5%, 28 89.7% versus 50%21 or 72.7% versus 52.6%,29 respectively. Finally, mutations in katG codon 315 have often been linked with a high level of isoniazid resistance (>4 mg/L).9,22,23,29 In this study, the katG 315 mutations were predominant both among MDR and isoniazid-monoresistant strains, yet among the latter they occurred about twice less frequently (72% versus 42%) and this difference was statistically significant (P<0.01). Another finding, which was consistent with the preceding ones, was that the MICs of isoniazid for katG 315 mutants were within the range of 1–10 mg/L, with median MICs of 2.5 mg/L for both MDR and isoniazid-monoresistant strains, corresponding to a moderate to high level of resistance. Of the 55 (36 MDR and 19 isoniazid-monoresistant) katG 315 mutants, all but 3 were catalase positive. This is again in agreement with previous observations that most

Discussion

Although an increasing number of studies have recently been published on the prevalence of mutations conferring isoniazid resistance in TB, only a few have involved ample strain collections or have investigated a wide array of genetic loci and over their full-length sequences. Even fewer studies have attempted to compare the distribution of isoniazid resistance-associated mutations between isoniazid-monoresistant, MDR and isoniazid susceptible strains. The discovery of mutations that would be detectable in the great majority of isoniazid-resistant strains, and never present in susceptible bacilli, would fulfill the criteria for an ideal marker for isoniazid resistance; whereas the identification of mutations that would exclusively occur in MDR strains, being absent in non-MDR isoniazid-resistant strains, could provide a marker for the MDR phenotype.

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Figure 1. Schematic representation of the distribution of mutations in the nine genetic loci investigated, with regard to MIC values of isoniazid. ins, insertion. Numbers in boxes shaded light grey or dark grey, below mutation designations, represent isolates with a particular mutation type and MIC of isoniazid. This figure appears in colour in the online version of JAC and in black and white in the print version of JAC.
isoniazid-resistant strains, exhibiting katG 315 mutation, preserve their catalase activity.6,26,30 The preponderance of katG 315 mutations, and their strong association with the MDR phenotype in particular, is explained by the low fitness cost of these mutations and the survival advantage they confer on isoniazid-resistant strains. Since katG 315 mutants are of equal virulence and transmission capacity to drug-susceptible strains,31,32 they are more likely to acquire new mutations and evolve into MDR strains. A question that remains elusive is, to what extent is the high frequency of katG 315 mutations a result of the transmission of isoniazid-resistant tuberculosis? This question fits into a larger debate, which provokes much controversy, about the transmission capacity of isoniazid-resistant or generally drug-resistant M. tuberculosis strains. Several studies have proven that isoniazid-resistant or MDR strains are far less likely to be clustered than susceptible strains.33–35 In contrast, large outbreaks of both isoniazid-monoresistant and MDR TB have been recorded.36,37 Similarly, contradictory findings exist with respect to katG 315 mutations. Some authors found them equally likely to be clustered as their susceptible counterparts,23 whereas others reported no clustering among katG 315 mutants, even though they represented >90% of all isoniazid-resistant strains examined.38 For the strains in this study, transmissibility was not evaluated except for MDR strains. Among these, the proportion of clustered cases came to ~22% (10 patients). All of the clustered cases harboured a Ser315Thr mutation, albeit more than two-thirds of the cases with this particular mutation were outside the clusters. Furthermore, a direct epidemiological link was clearly established for only four (9%) of the clustered cases.39 This suggests a relatively low transmissibility of MDR strains. Of these mutations, 17 seem to be associated with isoniazid-resistant strains only and had not been reported before. Eleven of these mutations were not accompanied by katG 315 or inhA promoter mutations and thus were likely to confer isoniazid resistance. However, to confirm unambiguously the involvement of these mutations in the development of isoniazid resistance is quite challenging, as they usually occurred in conjunction with other mutations in katG and/or other genetic loci. A strong association with isoniazid resistance could be established for four mutations, i.e. 18insA (frameshift), T302C (Leu101Pro), C752T (Thr251Met) and C874T (Pro292Ser), as they were all the only mutations to be found in isoniazid-resistant strains (Table 1). All but one (C752T, Thr251Met) were new and not described previously. Another three katG mutations (T547C, Phe183Leu; G771A, Met257Ile; and C1136T, Ala379Val) can also be correlated with isoniazid resistance, since they co-occurred with mutations (ndt T53C, Val188Ala; nat G619A, Gly207Arg; and mshA A332G, Asn111Ser) whose contribution to the isoniazid-resistant phenotype has been considered marginal or irrelevant.6,10,42

Among non-katG mutations, which occurred as the sole mutation in at least one isoniazid-resistant strain, were the T581C (Ile194Thr) substitution in the inhA structural gene, previously shown to be strongly associated with isoniazid resistance,43 as well as a 3 nt mutation (TCA→ AUG) in the oxyR-ahpC regulatory region and a termination mutation (C487T, Arg163STOP) in the ahpC gene, both so far unreported (Table 1). All these mutations that were present in isoniazid-resistant but not in isoniazid-susceptible strains, as evidenced from this work and literature data, and which occurred independently of katG 315 or inhA promoter mutations, are very likely to confer isoniazid resistance in tubercle bacilli. Consequently, these mutations may potentially serve as markers for the detection of isoniazid resistance in TB.

Although, most of the recent studies employ a multiple-loci approach when searching for mutations involved in isoniazid resistance, there are still strains with WT alleles at all loci analysed. The frequencies of such strains among the total resistant strains in studies that investigated similar loci (four to seven, including katG and promoters of inhA and ahpC), yet with different sequencing strategies (complete or partial gene sequencing) and in different strain samples, were within the range of 2.5%–35.6%.5,8,25,27,28 The percentage of resistant strains lacking any mutation was still high (10.5%), even when as many as 20 genes were screened.5 In this study, despite using a wide array of loci and their full-length sequence analysis, 14 (1 MDR and 13 isoniazid monoresistant) strains did not have mutations in any of the loci tested. Another three (isoniazid monoresistant) strains harboured mutations, as the only ones, considered not to be associated with isoniazid resistance. Hence, in 16 (32%) isoniazid-monoresistant strains (MIC range, 0.5–2.5 mg/L; mean MIC, 2.5 mg/L), genetic determinants of isoniazid resistance could not be determined.

Overall, out of 66 mutations detected in this study, 22 were not related to isoniazid resistance as they were found in isoniazid-susceptible strains. Among the remaining 44 mutations, there were 15 mutations that had previously been reported predominantly in isoniazid-resistant strains and 29 newly described mutations that occurred (in this study) only in isoniazid-resistant strains. Of these mutations, 17 seem to be associated with

The katG codon 315 and inhA promoter mutations represent two major mechanisms of isoniazid resistance. Given their overall relatively high frequency of detection, and the fact that they occur almost exclusively in isoniazid-resistant strains (high specificity), these mutations are generally considered as reliable markers of isoniazid resistance. The five most common mutations, including Ser315Thr in the katG gene and C—15T, A—16G, T—8C and T—15A alterations in the inhA promoter, can now be detected in a simple line probe assay using a commercially available test (GenoType MTBDR; Hain Life Sciences, Germany), whose accuracy has been extensively verified.61 Apart from those canonical mutations at katG 315 or the inhA promoter, a plethora of other mutations have been described and hypothesized to be associated, to different extents, with isoniazid resistance. In this study, of the 25 katG mutation types identified outside of codon 315, 9 had already been described in isoniazid-resistant but not isoniazid-susceptible strains. Of the remaining 16 katG mutations, 12 were non-silent, occurred in isoniazid-resistant strains only and had not been reported before. Eleven of these mutations were not accompanied by katG 315 or inhA promoter mutations and thus were likely to confer isoniazid resistance. However, to confirm unambiguously the involvement of these mutations in the development of isoniazid resistance is quite challenging, as they usually occurred in conjunction with other mutations in katG and/or other genetic loci. A strong association with isoniazid resistance could be established for four mutations, i.e. 18insA (frameshift), T302C (Leu101Pro), C752T (Thr251Met) and C874T (Pro292Ser), as they were all the only mutations to be found in isoniazid-resistant strains (Table 1). All but one (C752T, Thr251Met) were new and not described previously. Another three katG mutations (T547C, Phe183Leu; G771A, Met257Ile; and C1136T, Ala379Val) can also be correlated with isoniazid resistance, since they co-occurred with mutations (ndt T53C, Val188Ala; nat G619A, Gly207Arg; and mshA A332G, Asn111Ser) whose contribution to the isoniazid-resistant phenotype has been considered marginal or irrelevant.6,10,42

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isoniazid resistance as they were not accompanied by any other mutations known to confer isoniazid resistance. Six of these mutations were most likely involved in isoniazid resistance as they were the only mutations identified in an isolate or co-occurred with silent mutations and/or mutations proved to be irrelevant to isoniazid resistance (Table 1). The role of the remaining 12 mutations (out of the aforementioned 29 new mutations) in the development of isoniazid resistance could not be clearly established, as they co-occurred with other mutations known to confer isoniazid resistance.

To conclude, this study revealed 17 mutations, not previously reported, that might be of potential use as new surrogate markers of isoniazid resistance in TB. Twelve and five of these mutations were exclusively found in MDR and isoniazid-monoresistant strains, respectively. Their accuracy as predictive markers of MDR status and isoniazid monoresistance, respectively, requires further investigation on much larger strain samples and from different geographical settings. For detecting isoniazid resistance in TB, molecular approaches should still be a complement to rather than a replacement for conventional drug susceptibility profiling. This is supported by the lack of mutations in any of the nine genetic loci investigated in 18 isoniazid-resistant strains from this study. These strains should be subjected to further genetic analysis, preferably by using WGS technology, to disclose possibly new mechanisms involved in the causation of isoniazid resistance. Finally, one comprehensive and integrated world database listing mutations associated with isoniazid resistance should be erected to guide the development of new molecular markers for isoniazid resistance.

**Acknowledgements**

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**Table 1.** Selected mutations of potential use as markers for isoniazid resistance in *M. tuberculosis*

<table>
<thead>
<tr>
<th>Locus</th>
<th>Mutation</th>
<th>No. of isolates</th>
<th>INH MIC (mg/L)</th>
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<td>INH monoresistant</td>
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<tr>
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<td>1</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>A1180C&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>1</td>
<td>&gt;60</td>
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<tr>
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<tr>
<td><strong>inhA</strong></td>
<td>T581C&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>2</td>
<td>1</td>
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<tr>
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<td>G−48A&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>2</td>
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<td><strong>nat</strong></td>
<td>G518A</td>
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</table>

<sup>a</sup>A mutation that was the only mutation identified in an isolate or co-occurred with silent mutations and/or mutations not linked with isoniazid resistance, at any of the loci analysed.

<sup>b</sup>Previously described mutation.

<sup>a</sup>Included in this table are only mutations that were not accompanied by any other mutations known to be associated with isoniazid resistance.

<sup>b</sup>Ins, insertion; NA, not applicable; INH, isoniazid.
Transparency declarations

None to declare.

Supplementary data

Table S1 is available as Supplementary data at JAC Online (http://jac.oxfordjournals.org/).

References


