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Primers for Detecting *katG* Gene Mutations in Isoniazid-Resistant *Mycobacterium Tuberculosis*

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Abstract

Pulmonary tuberculosis is a disease caused by *Mycobacterium tuberculosis* infection, which is a health problem in Indonesia. Exploration of the treatment point of anti-tuberculosis drug resistance genes is very important for determining the appropriate gene-target markers of resistance. This study aimed to analyze the role of the *katG*/Rv1908c gene in *Mycobacterium tuberculosis* as a marker of isoniazid resistance, and to use mutation analysis data to design specific primers for dominant isoniazid-resistance mutations. We conducted descriptive research using a cross-sectional sampling method. The samples in this study were 10 MDR-TB isolates collected at the Health Laboratory and Calibration Testing Center of Central Java Province (Balapkes and Pak Prov Jateng). The research procedure included denaturing target primer sequencing of *Mycobacterium tuberculosis* DNA, DNA extraction, and PCR procedures. PCR was performed using BIONEER Exceller 96 with Gotaq Green Master Mix Kit reagents. PCR results were sequenced using the Sanger method at PT. Genetika Science. The Sanger sequencing results were analyzed using the BioEdit program to align the sequences. The spliced *Mycobacterium tuberculosis* DNA was analyzed using MEGA 10 software to identify mutation sites for comparison with the wild-type H37Rv and to determine the *katG* gene mutation site within the mapped region. The results of the DNA sequencing analysis using the Sanger sequencing method obtained that the *katG* gene mutation was dominated by Ser315Thr (80%) which was a single mutation, a double mutation at Ser315Thr+Ala221Asp (10%), a double mutation at Ser315Thr+Arg249Cys (10%), and the results of Primer F > 5'-GATCACCAGCGGCATCGAG-3' Primer R > 5'-CTCTTCGTCAGCTCCCACTC-3' and Primer R >5'-GATCACCACCGGCATCGAG-3' were able to amplify the Ser315Thr gene target as a detection primer. The conclusion is that the dominant mutation conferring isoniazid resistance is Ser315Thr, and the primers used to target the mutation work well.

Keywords: *Mycobacterium Tuberculosis, Primer PCR, Detection.*

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1. INTRODUCTION

Tuberculosis (TB) is the leading cause of death due to *Mycobacterium tuberculosis* infection, with 10 million cases of infection worldwide. 1.4 million deaths due to tuberculosis infection, and 465000 cases of drug-resistant tuberculosis. Ninety-three percent of TB cases are found in 16 countries, with the three largest contributing countries being India, Indonesia, and the Philippines (Lechevallier, 2004; Parums, 2021; World Health Organization, 2021; Dheda et al., 2022; Hatami et al., 2022).

Drug-resistant TB (DR-TB) cases across Indonesia over four years were 11.463 in 2019, 7921 in 2020, 8296 in 2021, and 12531 in 2022 (Ministry of Health, 2023). The number of MDR-TB cases in 2021 was 20500, increasing to 21900 in 2022 (Ministry of Health, 2023). The rising number of tuberculosis cases poses a challenge to global tuberculosis control, driven by the emergence of *Mycobacterium tuberculosis* resistance to anti-tuberculosis drugs. Delayed diagnosis and incomplete characterization of *Mycobacterium tuberculosis* resistant to anti-tuberculosis drugs have contributed to the transmission of cases. (Dlamini et al., 2019; Mirzayev et al., 2021; de Vos et al., 2021; Goossens et al., 2022).

The mechanism of action of isoniazid, or isonicotinoyl hydrazide (INH), is to inhibit the formation of mycolic acid, which is the main component for the formation of the cell wall of Mycobacterium tuberculosis. INH also inhibits several genes encoding the inhA reductase protein, which functions in mycolic acid biosynthesis to synthesize fatty acids (Prasad et al., 2021). INH was the first synthetic drug to act as a bactericide against *Mycobacterium tuberculosis* and was introduced as a TB treatment 40 years ago. Exposure to isoniazid in *Mycobacterium tuberculosis* can cause changes in the *katG* target gene, allowing bacteria to become resistant; changes in the drug-target gene allow isoniazid resistance to be encoded by the *katG* activator gene and the inhA-mabA target. (Garcia et la., 2019). Resistance of *Mycobacterium tuberculosis* to the anti-tuberculosis drug isoniazid is associated with mutations in the 315th amino acid locus of *katG*, the 15th amino acid of inhA-mabA, and the 8th amino acid of inhA-mabA (Valafar, 2021).

Gene mutations are an important etiologic factor in *Mycobacterium tuberculosis* resistance. Therefore, mutational studies of the *katG* gene are necessary to analyze *Mycobacterium tuberculosis* resistance patterns to anti-tuberculosis drugs, particularly isoniazid. The Ser315Leu mutation has the potential to be a marker of *Mycobacterium tuberculosis* resistance to the anti-tuberculosis drug isoniazid. This study aimed to analyze the role of the *katG*/Rv1908c gene in *Mycobacterium tuberculosis* as a marker of isoniazid resistance, and to use mutation analysis data to design specific primers for dominant isoniazid-resistance mutations.

2. RESEARCH METHOD

We conducted a descriptive study using a cross-sectional sampling method. Ethical clearance was obtained from the Health Research Ethics Committee of the Ministry of Health Polytechnic of Semarang, No. 853/EA/F.XXIII.38/2025. The samples were 10 isolates of multidrug-resistant tuberculosis collected from the Central Java Provincial Health Laboratory and Calibration Testing Center. *Mycobacterium tuberculosis* was cultured on Lowenstein-Jensen medium. The samples were decontaminated with 4% NaOH to kill bacteria other than *Mycobacterium tuberculosis*. The supernatant was discarded, and the tube was washed with phosphate-buffered saline. 100 µl of the sediment was inoculated onto Lowenstein-Jensen medium. Incubation was carried out at 37°C for 4-8 weeks with observation every 24 hours. To ensure additional safety during the extraction process, we inactivated *Mycobacterium tuberculosis* by immersing the samples in 10% formalin for 24 hours, then sterilized them by autoclaving at 121°C for 15 minutes.

DNA extraction was then performed using a modified spin column method. A 450 µL *Mycobacterium tuberculosis* suspension in PBS solution with a bacterial density of 2 Mcf was taken, and 50 µL of 4% NaOH dissolved in isopropanol was added. The mixture was then heated at 95°C for 5 minutes. The solution was transferred to a spin column centrifuge at 6.000 rpm, followed by washing with the DNA Washer at 6000 rpm. 50 µL of DNA eluol was added and centrifuged at 12000 rpm. The extraction results were stored at -20°C. PCR was performed using a BIONEER exceler 96 with the Gotaq Green Master Mix Kit reagent according to the procedure.

Table 1. PCR examination procedure

Component	Volume	Concentrarion
GoTaq Green Master Mix	12.5 µL	1X
Primer 10 µM	2.5 µL	1.0 µM
Primer 10 µM	2.5 µL	1.0 µM
DNA Template	5 µL	<250 ng
Nuclease- free water	25 µL	

Note: *µM: micromolar, µL: microliter, ng: nanogram*

To determine the location of the mutation gene that confers *Mycobacterium tuberculosis* resistance to isoniazid, targeting the *katG* gene, primers F: 5'-CTATTGGGGCAAGGAAGCCA-3' and R: 5'-TCTTCCAGGGTGCGAATGAC-3' were used to amplify the 982-bp fragment of the *Mycobacterium tuberculosis* H37Rv genome. The program used was Primer3Plus, and the analysis was performed using the in silico PCR amplification program. The *katG*/Rv1908c gene has a nucleotide length of 2223 bp, and a nucleotide sequence spanning 2153889–2156111. The. The targeted mutation spans nucleotides 2154230–2154990 or codons 310–370. An example of a change in nucleotide G to C at codon 315, amino acid serine to threonine, figure 3.2. The numbering system uses the Complete genome H37RV NC_0009623, with a gene length of 4411532 bp (Green et al., 2008; Mustafa, 2012; Vigo et al., 2019; Khan et al., 2023).

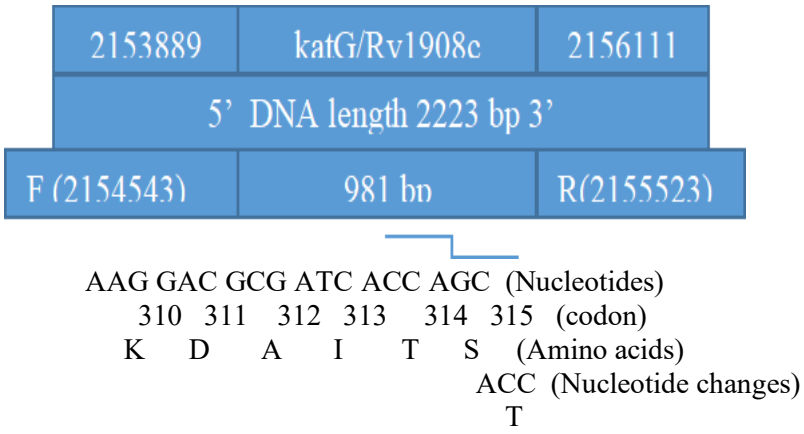

Figure 1. Location of the *katG* gene mutation and primer positions for DNA sequencing.

The primer is used to determine the mutation point. Data from dissertation research on frequently occurring mutation points are used to design detection primers. The primers we used were designed with Primer3Plus (https://www.primer3plus.com/index.html) using *Mycobacterium tuberculosis* H37Rv NC_00962 as the reference, with nucleotide positions 2155074 to 2155175 (102 bp), representing the complementary strand of the Rv1908c *katG* catalase peroxidase protein from 2153889 to 2156111.

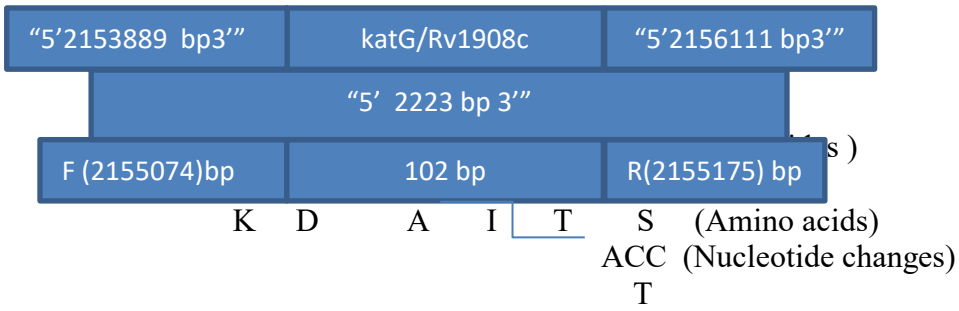

Figure 2. Primer locations for the target mutation of the *katG* gene.

Primer: F->5'-GATCACCAGCGGCATCGAG-3' Primer R -> 5'-CTCTTCGTCAGCTCCCACTC-3' and Primer F -> 5'-GATCACCACCGGCATCGAG-3' The PCR cycle was denatured at 95°C for 2 minutes, 40 cycles at 95°C for 30 seconds, 53°C for 30 seconds, and 72°C for 30 seconds. 0 C for 60 seconds, extension 72^0C for 5 minutes after the cycle is complete.

3. RESULTS AND DISCUSSION

The research results consist of two: the analysis of the Sanger sequencing results presented in table form, and the primary visualization of identification using electrophoresis.

Table 2. Results of point mutation analysis of the *katG* gene

Isolat	Mutasi gen *katG*			Total
	Ser315Thr (%)	Ser315Thr+Ala221Asp (%)	Ser315Thr+Arg249Cys (%)	
10 Isolat clinical	8	1	1	10 (100)
Total	8 (80)	1 (10)	1 (10)	10 (100)

Note: *Ala: Alanine, Cys: Cysteine, Asp: Aspartic Acid, Thr: Threonine, Arg: Arginine, Ser: Serine.*

The results of this study align with Catherine's research, which found that the Ser315Thr mutation is specific for isoniazid resistance at 94% (Vilchèze & Jacobs, 2015). Research in China showed that *katG* resistance reached 84,6% in the Xinjiang region (Li et al., 2024). The *katG* DNA strand, 2223 long, encodes the enzyme catalase peroxidase, which activates isoniazid into a toxic compound in bacterial cells. These primers are capable of amplifying the *KatG* S315T mutation region spanning codons 309 to 328. The isoniazid activation mechanism, activated by the mycobacterial catalase peroxidase *katG*, is called Rv1908c (Zhang et al., 2019). The radical amino acid isonicotinoyl (C6H4NO) will react with nicotinamide adenine dinucleotide (NAD+) to form the adduct INH-NAD (Saroja & Gopinathan, 1973; Snapper et al., 1990; Zhang et al., 1993; Zhang et al., 2019). This adduct binds and inhibits the activity of inhA (Jacobs et al., 1987; Zhang et al., 1993; Rozwarski et al., 1998; Wilming & Johnsson, 1999).

The enoyl-ACP reductase of *Mycobacterium tuberculosis*, Rv1484, is NADH-dependent, thereby inhibiting mycolic acid biosynthesis and leading to *Mycobacterium tuberculosis* cell death (Banerjee et al., 1994; Quémard et al., 1995; Mdluli et al., 1996; Wilming & Johnsson, 1999; Marrakchi et al., 2000). Wild-type *Mycobacterium tuberculosis* binds *katG* at amino acid S315, forming a hydrogen bond with the main chain Fe HEM and chain I317, while the mutant type of *Mycobacterium tuberculosis*,

katG 315T, obtains a weak hydrogen bond and hydrophobic interaction with HEM (Munir *et al.,* 2021).

The *katG* codon 309-328 region is frequently mutated, including *katG* S315T. The primer determination is expected to amplify this target and include targets outside this region to analyze possible mutations outside the frequently occurring mutation region. Enoyl acyl carrier protein reductase (ENR) inhA is a *Mycobacterium tuberculosis* enzyme that is the target of isoniazid in the treatment of tuberculosis.

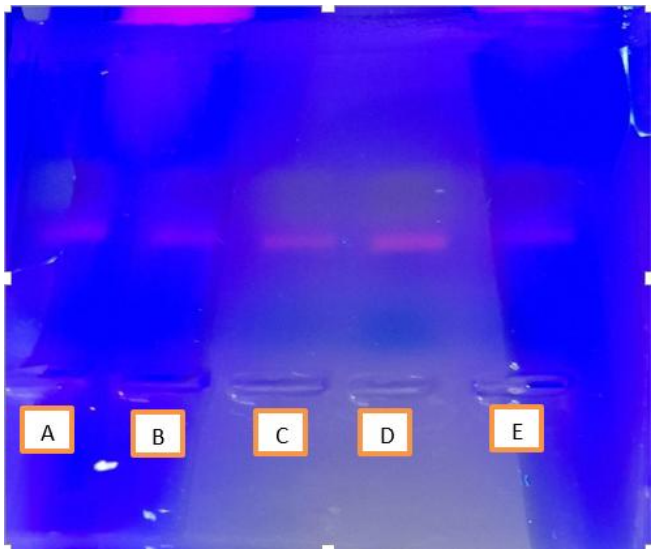

Figure 3. Primary test results with electrophoresis. A Primer *katG* 315, B Primer *katG* 315 mutant, C Primer *katG* 315, D Primer *katG* 315 mutant, E Primer *katG* 315, Samples A, B, C, D *Mycobacterium tuberculosis* is resistant to rifampicin due to mutations in the *katG* 315 gene; sample E is *Mycobacterium tuberculosis* H37Rv.

The results of the study showed that this primer was able to amplify the specified target and could be used as a specific primer candidate to determine isoniazid resistance caused by mutations in the *katG* 315 gene, this is part of the radical amino acid change isonicotinoyl (C6H4NO) will react with nicotinamide adenine dinucleotide (NAD+) to form an INH-NAD adduct (Saroja & Gopinathan, 1973; Snapper et al., 1990; Zhang et al., 1993; Zhang et al., 2019). The adduct binds and inhibits the activity of inhA (Jacobs et al., 1987; Zhang et al., 1993; Rozwarski et al., 1998; Wilming & Johnsson, 1999).

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The mechanism of isoniazid resistance in *Mycobacterium tuberculosis* involves nucleotide changes in the *katG* gene that can alter amino acid sequences, a process usually called a mutation. The mechanism of *Mycobacterium tuberculosis* resistance to the anti-tuberculosis drug isoniazid involves several possibilities, including changes in the drug target due to target gene mutations, overexpression of the drug target, impaired prodrug activation, and activation of efflux pumps (Miotto *et al.,* 2018). This study examines the mechanism *by which mutations in the katG gene confer* resistance to isoniazid in Mycobacterium tuberculosis.

Isoniazid is an anti-tuberculosis drug that inhibits the synthesis of mycolic acid. Activation of the mutant *katG* gene in isoniazid can render the drug ineffective at inhibiting mycolic acid synthesis. The Ser315Thr mutation is the most common in *katG*, namely the amino acid Serine (AGC) to Threonine (ACC). In addition, the amino acid

Serine can be converted to Asparagine, Arginine, Isoleucine, Glycine, or Leucine. Amino acid changes in *katG* alter the activity of the peroxidase and oxidase enzymes, causing *katG* to be unable to activate INH (Vilchèze & Jacobs, 2019).

The mechanism of isoniazid in *Mycobacterium tuberculosis* cells as a prodrug: it is a prodrug, does not affect eukaryotic cells, and is active only when activated by specific enzymes in pathogens that can cause death (Ghajavand *et al.,* 2019). The anti-tuberculosis drug isoniazid is activated by the mycobacterial catalase peroxidase *katG* (encoded by Rv1908c), which produces a hypothetical isonicotinoyl radical (Zhang et al., 2019). Inactivation of the katG gene in isoniazid-resistant mutants causes a radical change in isonicotinoyl, which reacts with nicotinamide adenine dinucleotide (NAD+) to form the INH-NAD adduct, thereby maintaining cell wall formation. (Saroja & Gopinathan, 1973; Snapper et al., 1990; Zhang et al., 1992; Zhang et al., 1993; Zhang et al., 2019). The adduct binds to and inhibits the activity of inhA (Jacobs et al., 1987; Rozwarski et al., 1998).

Isoniazid-resistant *Mycobacterium tuberculosis* has a nucleotide change in the *katG* gene that renders isoniazid inactive (Zhang et al., 1992). This study analyzes the mechanism of *Mycobacterium tuberculosis* resistance to the anti-tuberculosis drug isoniazid, which is caused by nucleotide changes in the *katG* gene. Isoniazid is an anti-tuberculosis drug that inhibits mycolic acid synthesis. Activation of isoniazid by mutant *katG* genes can render isoniazid unable to inhibit mycolic acid synthesis. The most common amino acid change at Ser315Thr is the change that changes the amino acid serine (AGC) to threonine (ACC). In addition, the amino acid serine can be replaced by the amino acids Asparagine, Arginine, Isoleucine, Glycine, or Leucine. Amino acid changes in *katG* alter the activity of the peroxidase and oxidase enzymes, causing *katG* to be unable to activate INH (Vilchèze & Jacobs, 2019).

Resistance testing using the Growth Indicator Tube (MIGT) liquid culture method takes 1–2 weeks, whereas the Lowenstein-Jensen (LJ) solid culture method takes 3–8 weeks. MIC measurements for isoniazid are categorized as susceptible with a MIC ≤ 0.2 mg/L, moderately resistant with a MIC ≤ 1.6 mg/L, moderately resistant with a MIC ≥ 12.8 mg/L, and high-level resistant with a MIC ≥ 19.2 mg/L. The most common mutation conferring moderate resistance to isoniazid is a change in katG from Ser315 to Thr. High-level resistance to isoniazid is conferred by a mutation in *katG* (Ser315Thr) combined with a mutation in inhA (C/T-15) (Lempens et al., 2018). The results of this study can be used as an alternative rapid test for detecting isoniazid-resistant *Mycobacterium tuberculosis,* with the katG gene as the primary target.

4. CONCLUSION

Conclusions: Isoniazid-resistant *Mycobacterium tuberculosis* had a dominant mutation at ser315thr. This result is the basis for making specific primers for identifying mutations in the *katG* gene: Primer F >5'-GATCACCAGCGGCATCGAG-3', Primer R> 5'-CTCTTCGTCAGCTCCCACTC-3' and Primer F > 5'-GATCACCACCGGCATCGAG-3'.

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