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Comparative analysis of analytical sensitivity of qPCR methods for detecting isoniazid-resistant tuberculosis using SYBR Green and TaqMan Probe

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Abstract: Tuberculosis (TB) is an infectious disease caused by *Mycobacterium tuberculosis* and remains one of the leading causes of death worldwide. The increasing prevalence of TB strains resistant to multiple antibiotics, including isoniazid, highlights the urgent need for rapid and accurate molecular diagnostic methods. One such method is quantitative polymerase chain reaction (qPCR). This study aimed to compare the analytical sensitivity of qPCR assays for detecting isoniazid-resistant *M. tuberculosis* using SYBR Green and TaqMan Probe chemistries. Analytical sensitivity was evaluated using DNA from isoniazid-resistant *M. tuberculosis* carrying the S315G mutation, which was serially diluted up to 10^{-9}. Primary data were analyzed using probit regression analysis. The results showed that the analytical sensitivity of the SYBR Green method was 1.5045×10^{-3} ng/µL, while that of the TaqMan Probe method was 1.0205×10^{-3} ng/µL. These findings indicate that the qPCR TaqMan Probe method is approximately 1.47 times more sensitive than the SYBR Green method. However, further studies evaluating additional validation parameters are required to determine the most suitable method for routine diagnostic application.

Keywords: Isoniazid; Method Validation; qPCR; Sensitivity; Tuberculosis.

INTRODUCTION

Drug-resistant tuberculosis (DR-TB), particularly isoniazid, is still a serious public health concern in many countries around the world and poses a threat to the control of tuberculosis. According to estimations, the incidence of DR-TB cases in Indonesia is expected to be 24,000 or 8.8/100,000 people, accounting for 2.4% of all new TB patients and 13% of cured TB patients[1]. In Indonesia, the treatment of DR-TB patients has been in place since 2009. The outcomes of treating DR-TB patients between 2009 and 2017 indicate a trend toward a decline in treatment success rates, an increase in treatment discontinuations, and a rise in treatment-related deaths[2]. Therefore, Indonesia is still working to improve the effectiveness and efficiency of DR-TB diagnosis.

Mycobacterium tuberculosis (MTB) resistance towards drugs results from spontaneous chromosomal mutations. Resistance to isoniazid can be caused by the patient not complying with the doctor's/health worker's recommendations, not regularly consuming the anti-tuberculosis drug (ATD) combination, unilaterally

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stopping the treatment prematurely, or the patient having impaired drug absorption. Treatment for tuberculosis (TB) results in the selective suppression of the MTB bacterial population, killing susceptible MTB bacteria while mutant populations proliferate and develop acquired resistance to ATD. Patients who have undergone TB therapy for at least a month, including those who have failed treatment, relapsed, or returned after stopping treatment, are at risk of developing resistance among previously treated patients. These individuals may develop resistance bacteria while receiving therapy, or they may get a primary or reinfection from a person who already has resistant tuberculosis[2,3].

The process of confirming the diagnosis of DR-TB requires several stages and a long time. Patients suspected of TB must undergo BTA microscopy and TB molecular rapid tests. If the result is positive for rifampicin-sensitive MTB, the patient must undergo first-line TB treatment before isoniazid resistance can be determined. Meanwhile, if rifampicin-resistant MTB is positive, the patient needs to start DR-TB treatment and needs further examination of first- and second-line ATD, which takes months. Owing to the long diagnostic pathway, healthcare facilities and researchers are trying to cut/speed up the DR-TB diagnostic pathway to make it more effective and efficient. Hence, a diagnostic kit for DR-TB is still under development[2–4].

Several laboratory tests can be used to detect TB, ranging from conventional methods such as microscopic examination and culture of bacteria from clinical specimens, to molecular detection-based methods such as GeneXpert, Line Probe Assays (LiPAs), and Polymerase Chain Reaction (PCR). PCR and quantitative PCR (qPCR) are currently the most popular methods because they are relatively faster than the culture method and can use all types of clinical specimens obtained from patients with TB[5].

The commonly used qPCR detection methods are SYBR Green and TaqMan probes. SYBR Green is a dye that intercalates with double-stranded DNA. This method is cheap and easy to administer, but it is highly dependent on the specificity of the primer to the targeted gene and the need for melting curve analysis. Meanwhile, TaqMan Probe is a probe-based method that has reporter dyes and quencher dyes so that it is relatively able to avoid non-specific amplicons although it is relatively more expensive and requires different probe designs for each different target gene[6,7]. Currently, the testing is focused on qPCR for isoniazid-resistant TB, for which the kit is still under development. Therefore, method validation of kit development is required.

Method validation is used to ensure the accuracy and precision of an analytical method or instrument. Validation studies were designed to provide evidence that the analytical procedure met its objectives. Analytical sensitivity or limit of detection (LoD) is one of the parameters that needs to be tested when performing method validation. Analytical sensitivity is the ability of a test to detect an analyte. In direct detection, analytical sensitivity is expressed as the lowest infectious dosage, lowest number of genome copies, plaque-forming units, colony-forming units, and lowest number of agents that can still be detected and separated from the background matrix[8]. Analytical sensitivity is very important to study to determine when or how many isoniazid-resistant TB bacteria can be detected through qPCR testing[9].

Although both SYBR Green and TaqMan qPCR methods are both widely used, comparative sensitivity studies of these methods for detecting isoniazid-resistant TB, particularly the S315G mutation using clinical isolate from a heavy TB burden country like Indonesia, remain limited. This study aimed to bridge this gap by comparatively analyzing of the analytical sensitivity of the two qPCR methods for detecting isoniazid-resistant Mycobacterium tuberculosis using S315G-targeted primers and probes.

MATERIAL AND METHOD

Materials

The research unit used was a pooled sputum specimen collected confirmed to be positive for isoniazid-resistant *Mycobacterium tuberculosis* by BD MAX PCR examination. The materials used in this study were Viral Nucleic Acid Extraction Kit (GeneAid), GoTaq Master Mix Kit (Promega), GoTaq Probe Master Mix Kit (Promega), S315G Target Gene Primer and Probe Set, NaOH (Merck), Absolute Ethanol (Merck), Nuclease-free water.

DNA Isolation (Spin Column Method) and DNA Concentration Measurement

The mucopurulent sputum was pipetted to a volume of 200 µL, placed into a 1.5 mL microtube, and then diluted using 4% NaOH solution in a 1:1 ratio. Next, the specimen mixture was spun at 15000 rpm for 2 min, and the supernatant was discarded. The procedure was carried out according to the GeneAid Viral Nucleic Acid Extraction Kit. The concentration of extracted DNA was measured using a NanoDrop Nanophotometer NP80 Spectrophotometer. Extracted DNA was stored frozen at -20˚C until further analysis.

Serial DNA Dilution

Isoniazid-resistant *M. tuberculosis* genomic nucleic acids extracted from sputum were serially diluted 10-times fold for 10 variations in a final volume of 50 µL each.

Quantitative-PCR Process

The target amplified in this study was isoniazid-resistant *M. tuberculosis* carrying the KatG S315G mutation gene. The master mix used in the SYBR Green method was Go Taq qPCR Promega. The master mix was prepared with a final concentration of Go Taq qPCR of 1X, forward primer and reverse primer of 200 nM in 20 µL. The reaction mix total volume was 20 µL without template. The DNA template was added at a volume of 5 µL. The total reaction volume for 1 SYBR Green qPCR cycle was 25 µL. The PCR process was conditioned for 1 cycle for the pre-denaturation phase, at 95^{O}C, for 2 min; denaturation phase for 40 cycles, at 95^{O}C, for 15 s; and annealing extension phase for 40 cycles, at 56^{O}C, for 1 min.

For the TaqMan probe method, Go Taq Probe qPCR Promega was used as the master mix. The master mix was made with a volume of 15 µL, with a final concentration of Go Taq Probe qPCR of 1X, forward primer and reverse primer 400 nM, and probe 300 nM. The DNA template was added at a much of 2.5 µL. The total reaction volume for 1 TaqMan qPCR cycle was 17.5 µL. PCR conditions for this method were 1 cycle of pre-denaturation phase, temperature 95^{O}C, for 2 min; 50 cycles of denaturation phase, temperature 95^{O}C, for 15 s; and 50 cycles of annealing extension phase, temperature 62^{O}C, for 1 min.

Data Analysis

A logarithmic regression curve was created by plotting the known DNA concentration or dilution against the Ct value of each serial dilution to establish the confidence level of qPCR assay results. Through the probit regression test (Microsoft Excel), the lowest concentration that still gave an R^2 value ≥ 0.950 in the qPCR assay of SYBR Green and TaqMan Probe method was set as the analytical sensitivity value.

RESULTS AND DISCUSSION

DNA Target Preparation

In this study, sputum that was confirmed to be positive for isoniazid-resistant *M. tuberculosis* was extracted and henceforth referred to as DNA target. After obtaining the DNA target, its concentration was calculated using a NanoDrop

spectrophotometer at a wavelength of 260 nm. The concentration of the DNA target was 20.55 ng^1uL^{-1}.

The research was continued by performing serial dilutions 10-times fold against the DNA Target with 10 variations in concentration (10^0 – 10^9). qPCR was performed using SYBR Green and TaqMan Probe methods.

Analytical Sensitivity of SYBR Green Method

In the qPCR SYBR Green method, 60 data points were successfully obtained from triplicates of 10 concentration variations for two days. Based on obtained data, a regression curve of dilution against the Ct value was constructed and observed for each replication until the dilution of the curve still yielded an R^2 value ≥ 0.950. As presented in **Table 1**, results showed that up until the 10^4 dilution (concentration 2.055 x 10^{-3} ng^1uL^{-1}), all replicates (1–6) produced R^2 value ≥ 0.950. Meanwhile, at dilution 10^5 (concentration 2.055 x 10^{-4} ng^1uL^{-1}), only four replicates gave an R^2 value ≥ 0.950**.** At dilution 10^6 (concentration 2.055 x 10^{-5} ng^1uL^{-1}), only one replicate gave an R^2 value ≥ 0.950.

Table 1. Mean ±SD value of Ct and R^2 value of qPCR SYBR green method.

Dilution	Mean ±SD value of Ct and R^2 value qPCR SYBR Green Method			
	Ct		R^2 value	
10^0	22.25	±0.648	N/A	N/A
10^1	25.44	±1.078	1.00	±0.000
10^2	28.29	±0.881	0.99	±0.013
10^3	31.36	±0.546	0.99	±0.009
10^4	32.65	±1.016	0.98	±0.008
10^5	33.48	±1.208	0.95	±0.019
10^6	33.78	±1.287	0.91	±0.027
10^7	33.74	±1.774	0.86	±0.045
10^8	34.47	±2.107	0.83	±0.059
10^9	33.80	±0.958	0.77	±0.022

Furthermore, the proportion of the number of replicates that provided R^2 ≥ 0.950 to the total replicates was calculated to obtain the probit value. The probit value is the result of converting the proportion to probability units on a standard-deviation scale. The probit value can be determined using the Finney table. In determining analytical sensitivity, the probit point used was C_{95}, which was defined as the concentration at which 95% of the samples containing the analyte concentration were positive (in this study, positive means R^2 ≥ 0.950).

Table 2. Probit value of analytical sensitivity determination of SYBR green method.

Concentration (ng^1uL^{-1})	Log10 Concentration	N Total	N $R^2 \geq 0.950$	Proportion	Probit
2.055 x 10^1	1.3128	6	6	1.000	N/A
2.055 x 10^0	0.3128	6	6	1.000	N/A
2.055 x 10^{-1}	-0.6872	6	6	1.000	N/A
2.055 x 10^{-2}	-1.6872	6	6	1.000	N/A
2.055 x 10^{-3}	-2.6872	6	6	1.000	N/A
2.055 x 10^{-4}	-3.6872	6	4	0.667	5.431
2.055 x 10^{-5}	-4.6872	6	1	0.167	4.033
2.055 x 10^{-6}	-5.6872	6	0	0.000	N/A
2.055 x 10^{-7}	-6.6872	6	0	0.000	N/A
2.055 x 10^{-8}	-7.6872	6	0	0.000	N/A

The Log10 Concentration values (X) in **Table 2** were plotted against the probit values (Y) to obtain the linear equation shown in **Figure 1**. The curve presented in **Figure 1** shows a strong positive correlation which indicate that as

the Log10 value increases, so does the probit value. The probit value of C_{95} is 6.64. This value was entered as the Y value into the line equation y = 1.398x + 10.586 to obtain a Log10 Concentration value of -2.8226 and converted to a concentration of 0.0015045 or 1.5045 x 10^{-3} ng^1uL^{-1}. This value was the lowest target DNA concentration that provided an R^2 value of ≥ 0.950.

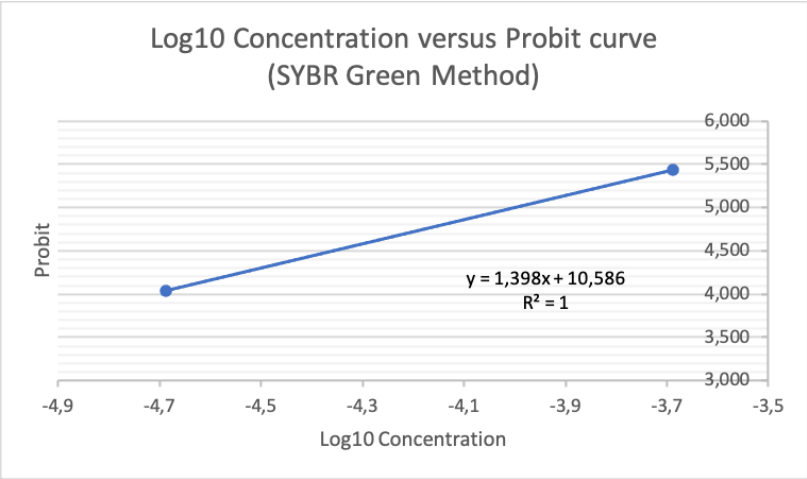

Figure 1. Log10 concentration against probit curve for SYBR Green Method.

Analytical Sensitivity of TaqMan Probe Method

A total of 60 data points were collected from triplicates of 10 concentration variations over two days. As shown in **Table 3**, the Ct value generally continued to increase until dilution 10^4, then the Ct value of some replicates began to be unstable at dilution 10^5–10^7 and continued to experience instability until the last dilution (10^9).

Table 3. Mean ±SD value of Ct and R^2 value of qPCR TaqMan Probe method

Dilution	Mean ±SD value of Ct and R^2 value qPCR TaqMan Probe Method			
	Ct		R^2 value	
10^0	19.76	±0.147	N/A	N/A
10^1	21.42	±0.377	1.00	±0
10^2	22.97	±0.497	0.99	±0.011
10^3	24.90	±0.879	0.99	±0.011
10^4	25.95	±0.246	0.98	±0.016
10^5	26.46	±2.105	0.88	±0.228
10^6	26.90	±2.365	0.82	±0.295
10^7	24.55	±2.941	0.56	±0.293
10^8	20.65	±1.645	0.14	±0.109
10^9	18.20	±0.973	0.00	±0.003

Almost all replicates experienced a drastic decrease in R^2 values with large dilution variations. Similar to the SYBR green Method results, every replication until the 2.055 x 10^{-4} ng^1uL^{-1} concentration gave R^2 value ≥ 0.950. However, 1 replication at the 10^5 dilution could not provide the minimum R^2 value of 0.950. The R^2 value kept dropping as the concentration continued to decrease. After that, the probit value was calculated as shown in **Table 4**.

Table 4. Probit value of analytical sensitivity determination of TaqMan probe method.

Concentration (ng^1uL^{-1})	Log10 Concentration	N Total	N $R^2 \geq 0.950$	Proportion	Probit
2.055×10^1	1.3128	6	6	1.000	N/A
2.055×10^0	0.3128	6	6	1.000	N/A
2.055×10^{-1}	-0.6872	6	6	1.000	N/A
2.055×10^{-2}	-1.6872	6	6	1.000	N/A
2.055×10^{-3}	-2.6872	6	6	1.000	N/A
2.055×10^{-4}	-3.6872	6	5	0.883	5.967
2.055×10^{-5}	-4.6872	6	3	0.500	5.000
2.055×10^{-6}	-5.6872	6	1	0.167	4.033
2.055×10^{-7}	-6.6872	6	0	0.000	N/A
2.055×10^{-8}	-7.6872	6	0	0.000	N/A

After plotting the Log10 Concentration value against the probit value in **Table 4**, a linear regression line equation was obtained, as shown in **Figure 2**. The presented regression curve for this method has 3 data points with a strong positive correlation between the Log10 concentration and the probit value. To acquire the lowest DNA concentration, the probit value of C_{95} (6.64 as Y) was entered into the equation y = 0.967x + 9.5325, and a Log10 value of -2.99121 was obtained. After conversion, the lowest DNA target concentration value that still gave $R^2 \geq 0.950$ was 0.0010205 or 1.0205×10^{-3} ng^1uL^{-1}.

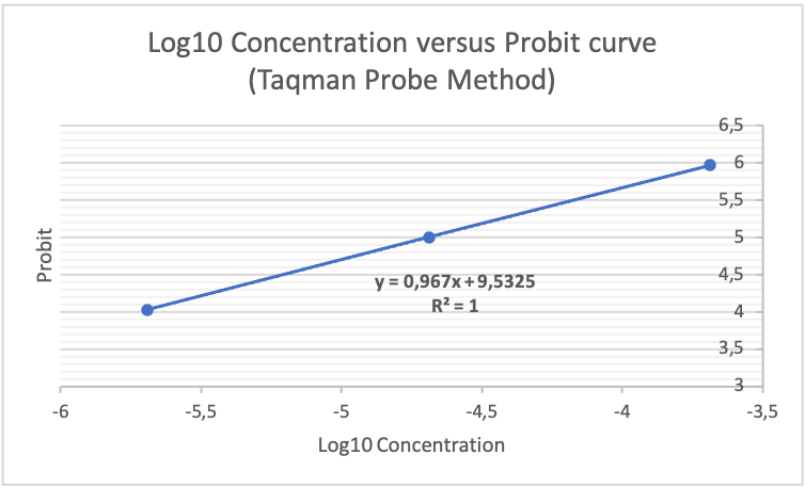

Figure 2. Log10 concentration versus probit curve for TaqMan probe method.

Resistance to ATD is mainly due to genetic mutations in *M. tuberculosis*. The inadequacy of the drug around the bacteria induces bacterial resistance and results in the spread of resistant bacteria[10]. Resistance to isoniazid (INH) is generally caused by mutations in KatG gene.

KatG gene is located in the sequence between 2,153,445 - 2,156,555 of the *M. Tuberculosis* genome. The KatG gene is 2,223 bp in length and is responsible for producing the catalase-peroxidase protein. A mutation in the codon of KatG causes a change in the catalase-peroxidase protein, resulting in the formation of INH-resistant *M. tuberculosis*. The most common mutation is located in codon 315[11].

The most common amino acid change is located at codon 315Ser. This was reinforced by the results of a study, which revealed that 56% of mutations occurred at the KatG315 locus, 20% of mutations occurred at the KatG463 locus, and 24% occurred without mutations from 25 INH-resistant strains[12]. The most prevalent mutation at codon 315Ser in South East Asia is substitution[13]. The

prediminant type was Ser315Thr (AGC → ACC), although there were also mutations of Ser315Gly (AGC → GGC), Ser315Arg (AGC → AGA or AGC → CGC), Ser315Ile (AGC → ATC), Ser315Asn (AGC → AAC), Ser315Thr (AGC → ACA)[14-16].

The S315G primer was used in this study. The Ser315Gly mutation (S315G) is the second most common type of mutation after Ser315Thr among other 315Ser types[17]. The S315G mutation is a missense mutation, which is a change in the genetic code that causes the amino acids associated with the polypeptide chain to change. In S315G, the amino acid that should be serine changes to the amino acid glycine. Codon 315 in MTB should be “AGC” but turns into “GGC”. From these changes, it can be concluded that the S315G mutation was caused by a transition event. Transitions are changes between purine bases (A and G) or between pyrimidine bases (C and T).

This study aimed to compare the analytical sensitivity or limit of detection (LoD) of isoniazid-resistant *Mycobacterium tuberculosis* testing using qPCR with the SYBR Green method and the TaqMan Probe method. SYBR Green is a specific dye for double-stranded DNA (dsDNA) and is most often used in qPCR. SYBR Green is an asymmetric cyanine dye that mostly binds to dsDNA, does not bind to single stranded-DNA (ssDNA), and does not consider the nucleotide sequence. During the extension stage of PCR, SYBR Green bonded to each new dsDNA copy as the target nucleotide sequence was amplified. At a maximum wavelength of 520 nm, SYBR Green's emission spectra is similar to that of fluorescence. It may be activated by blue light at a wavelength of 480 nm. There was a greater fluorescence signal because the dye coupled to the dsDNA had 1000 times more fluorescence than the unbound dye. When polymerization is occurring, this causes a rise in the fluorescence signal that can be seen in real time, and it decreases when the DNA is denatured[18].

Fluorescence measurements were therefore carried out at the end of each PCR cycle's elongation phase. The absence of qPCR probes makes this approach less costly. Its primary flaw is that the primers alone define its specificity, therefore during optimization, the possibility of non-specific amplification products must be considered[19].

TaqMan probes are 18-22 base pairs long oligonucleotides doubly labeled with a fluorophore reporter at the 5' end (reporter dye) and a quencher at the internal or 3' end (quencher dye). TaqMan belongs to a type of hydrolysis probe. Similar to PCR, qPCR testing using the TaqMan method requires a pair of primers that are specific to the target gene. In addition to a pair of primers, a probe that can hybridize to the target gene is required. Thus, the polymerization process takes place on the target gene that has attached or hybridized with the probe. Following the denaturation step, the probe hybridizes to the target when the right amplicons are amplified, and it stays hybridized until the polymerase replaces its 5' end. Subsequently, the enzyme breaks down the probe, releasing the quencher from the fluorophore, causing it to fluoresces[20].

The resulting increase in fluorescence was proportional to the amount of amplified PCR product. As with polymerases, probe cleavage occurs only when the probe is hybridized with the complementary strand. Temperature conditions during the PCR polymerization phase must be adjusted or optimized to ensure probe binding[21].

After all the data were collected, data processing and statistical testing were performed. To determine analytical sensitivity, a logarithmic regression curve was created for each replicate and it was determined until dilution that could still provide an R^2 value ≥ 0.950. This value was chosen because it was expected that the inaccuracy in the results would be no greater than 5%. Probit regression analysis was used for the statistical analysis as it is a generally used method to evaluate

the analytical sensitivity/LoD for quantitative test such as qPCR[22]. Similar studies for validating qPCR method for detecting MTB also used probit analysis to calculate the method's LoD[22,23]. For analytical sensitivity studies, 95% (C_{95}) was used as the point. C_{95} represents the concentration at which 95% of samples contain the concentration of the analyte test positive (in this study, the R^2 value ≥ 0.950).

Things to note when probit analysis is the concentration value tested, the number of samples per concentration that are positive, and the total number of samples tested per concentration. The percentage response at each concentration was converted into a probit. The log10 of each concentration was calculated and a graph of the log concentration against the probit was plotted. The C_{95} value was entered into the line equation so that the log value could be converted into a concentration value[24–26].

The results of this study showed that the TaqMan probe method is more sensitive than the SYBR Green method. Previous studies have shown that TaqMan Probe qPRC exhibits higher sensitivity than SYBR Green qPCR in detecting various genes from various specimens[27–30]. Other than that, Ayalew et al. reported a limit of detection for detecting *M. tuberculosis* complex targeting IS1081 using TaqMan Probe qPCR method in saliva[31]. Our findings are consistent with these results, confirming that TaqMan Probe offers a superior analytical sensitivity (1.47-fold) in detecting the S315G mutation.

TaqMan Probe has higher sensitivity because in addition to using primers that are designed specifically complementary to the DNA target sequence, the TaqMan method also uses probes that are designed specifically and complementary to the DNA target sequence. Thus, the results of the increased fluorescence using the TaqMan method are only specific amplicons of the desired DNA target. This is different from the SYBR Green method, in which the dye intercalates against all double-stranded DNA amplicons during qPCR. Therefore, the background produced by the SYBR Green method was greater than that produced by the TaqMan method because the SYBR dye can intercalate against non-target templates.

Although the TaqMan method is more sensitive than the SYBR Green method, there are still many molecular examinations using the SYBR Green method, such as the detection of antibiotic-resistant *Streptococcus pneumoniae* genes[32], SARS CoV-2[33], *Trypanosoma cruzi*[34], PED virus[35], *Angiostrongylus cantonensis*[36], *Bartonella sp.*[37], Tilapia Lake virus[38], Laryngotracheitis virus[39], *E. coli*[40], etc.

CONCLUSION

Based on the results and discussion, it can be concluded that the analytical sensitivity value in qPCR of the TaqMan Probe method is 1.47 times more sensitive than that of the SYBR Green method in detecting isoniazid-resistant *Mycobacterium tuberculosis*. To determine which method is better for the examination, further research needs to be conducted on other method validation parameters.

AUTHORS' CONTRIBUTIONS

Fusvita Merdekawati: Conceptualization, Validation, Project Administration**. Betty Nurhayati:** Conceptualization, Supervision, Writing-Review & Editing**. Muhammad Raihan Suryaman:** Formal Analysis, Investigation, Writing-Original Draft**. Aditya Juliastuti:** Resources, Data Curation**. Acep Tantan Hadiana:** Validation, Investigation **. Sonny Feisal Rinaldi:** Validation, Visualization.

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DATA AVAILABILITY STATEMENT
Derived data supporting the findings of this study are available from the corresponding author on request.

DISCLOSURE STATEMENT
The views and opinions expressed in this article are those of the authors and do not necessarily reflect the official policy or position of any affiliated agency of the authors. The data is the result of the author's research and has never been published in other journals.

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