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Assessment of Analytical Specificity in qPCR: SYBR Green and TaqMan Probe Methods for Isonazid-Resistant *Mycobacterium tuberculosis*

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ABSTRACT

Background & Objective: *Mycobacterium tuberculosis*, often referred to as *M. tuberculosis*, is an infectious pathogen that is responsible for causing tuberculosis (TB), a prevalent condition that is a leading cause of death globally. The spread of tuberculosis bacteria that are resistant to certain medicines, such as isoniazid, is currently on the rise. Therefore, molecular testing, such as quantitative polymerase chain reaction (qPCR), is needed to rapidly and reliably identify isoniazid-resistant tuberculosis germs.
Method: The purpose of this study was to assess the analytical specificity of TaqMan Probe and SYBR Green qPCR techniques for the detection of isoniazid-resistant tuberculosis. This study was descriptive and quantitative. Analytical specificity was ascertained using MTB DNA with the S315G mutation spiked with E. coli DNA. A paired t-test was used to assess the primary data.
Result: The results show that the analytical specificity values based on the significance of the paired t-test for the SYBR Green and TaqMan Probe methods were 0.398 and 0.790, respectively.
Conclusion: Based on these results, it can be concluded that the analytical specificity of the TaqMan Probe qPCR method was 1.99 times greater than that of the SYBR Green method.

Keywords: Tuberculosis; qPCR; Specificity; Method Validation.

INTRODUCTION

Resistance to anti-tuberculosis drugs (ATDs) is mainly attributed to suboptimal treatment owing to poor medication adherence among patients with tuberculosis (TB), particularly among those who do not complete their prescribed course of drugs (Kebede, 2019; Santra et al., 2021). Patients with drug-resistant TB can spread the disease to those around them, making it challenging to implement TB control programs because of the presence of *Mycobacterium tuberculosis* (*M. tuberculosis*) that is resistant to ATDs, known as Multidrug-Resistant Tuberculosis (MDR-TB) (Singh et al., 2020). MDR-TB is caused by *Mycobacterium tuberculosis*, which exhibits resistance to two anti-tuberculosis drugs, isoniazid and rifampicin (Micheni et al., 2021; Traoré et al., 2023). In 2018, the World Health Organization (WHO) reported an increase in the number of multidrug-resistant tuberculosis (MDR-TB) cases, accounting for 7.4% of new tuberculosis (TB) cases and 11.4% of retreated TB cases. MDR-TB has been classified by the WHO as one of the top ten global health threats (Cocozza et al., 2020).

TB diagnosis proceeds in multiple stages, including microscopic examination, culture, and molecular-based tests such as GeneXpert, Line Probe Assay (LiPAs), and Polymerase Chain Reaction (PCR) (Günther et al., 2022; Nakate et al., 2019; Sethi et al., 2022). GeneXpert is limited to identifying M. tuberculosis and rifampicin resistance (Opota et al., 2019; Patel et al., 2020). In contrast, GenXpert extensively drug-resistant TB (XDR-TB) and culture tests are required to diagnose patients with isoniazid (INH) monoresistance (Cho et al., 2022). If the patient had undergone six months of treatment and a microscopic examination revealed the presence of M. tuberculosis germs, this investigation may still be performed. The delayed identification of isoniazid resistance can lead to a delay in treatment. Therefore, accurate and timely detection is crucial to prevent treatment failure and relapse in isoniazid-resistant TB patients (Butnaru et al., 2023). To expedite the diagnostic procedure, diagnostic kits that concurrently detect M. tuberculosis and isoniazid resistance using Real-Time or Quantitative PCR (qPCR) have been developed.

qPCR testing is preferred over culture methods because of its ability to utilize any kind of clinical specimen from patients with TB and its relative speed, sensitivity, and specificity (Artika et al., 2022). It is anticipated to accelerate the diagnosis of drug-resistant cases by using a single procedure. TaqMan probes and SYBR Green are two popular qPCR methods. Interaction of SYBR Green with double-stranded DNA. This approach is simple to use and economical; however, it requires melting curve analysis and depends significantly on primer specificity. TaqMan probes, on the other hand, are probe-based techniques that use reporter and quencher dyes to prevent nonspecific amplicons. However, this method is costly and requires unique probe designs for every target gene (Baschien & Carl, 2020; Zhang et al., 2020). Currently, the focus is on quantitative polymerase chain reaction (qPCR) for isoniazid-resistant tuberculosis (TB). Currently, diagnostic kits are in the validation stage for this method.

Validation is confirmation through examination and the provision of objective evidence that specific requirements for a particular use can be consistently met. This includes determining accuracy, precision, reference intervals, analytical sensitivity, and analytical specificity (Agalloco, 2021; Chung et al., 2021). Analytical specificity assesses whether the qPCR method is specific to patients infected with isoniazid-resistant TB, ensuring high accuracy and minimizing errors such

as false negatives and false positives. Therefore, it is essential to determine the analytical specificity of SYBR Green and TaqMan Probe methods for detecting isoniazid-resistant *Mycobacterium tuberculosis* using quantitative Polymerase Chain Reaction.

OBJECTIVE

The purpose of this study was to assess the analytical specificity of TaqMan Probe and SYBR Green qPCR techniques for the detection of isoniazid-resistant tuberculosis.

METHOD

This study used a descriptive quantitative research design with independent and dependent variables is used in this study. The comparison of the TaqMan probe method and the SYBR Green method, each with three distinct concentrations of interfering DNA, serves as the study's independent variable. In this study, the analytical specificity attained using both approaches was the dependent variable Sample.

The sputum, which has been verified to be positive for isoniazid-resistant M. TB, serves as the research unit. Twenty-four trials were carried out with one baseline DNA concentration and three distinct concentrations of interfering DNA (spike samples). SYBR Green and TaqMan Probes were the two qPCR reading techniques used to examine each concentration, which were tested in triplicate.

Variable

The independent variable in this study was the comparison between the SYBR Green and TaqMan Probe methods. Both methods were tested by adding interfering DNA from Escherichia coli at three different concentrations. The dependent variable in this study was the analytical specificity, as measured by the final CT values.

Data Collection Process

The 24 key data points used in this investigation are CT values from triplicate baseline specimens and triplicate samples with three distinct interfering DNA amounts that were obtained by both qPCR testing techniques. The analytical specificity of these raw data was subsequently achieved through processing.

DNA extraction was performed on *M. tuberculosis* genomic DNA that was resistant to isoniazid to confirm analytical specificity. The concentration of the extracted DNA was then determined. The interfering DNA was also isolated from the E. coli genome. Subsequently, a qPCR master mix was prepared using SYBR Green and TaqMan Probe techniques. The master mixes for SYBR Green and TaqMan Probe had already undergone compositional optimization. Next, M. tuberculosis DNA was added to the master mix as the baseline DNA and as the interfering DNA by mixing *M. tuberculosis* DNA with *Escherichia coli* DNA. Baseline DNA to interfering DNA ratios were 5:1, 5:3, and 1:1. Next, the KatG 315 gene was amplified using both techniques, and the amplification curves were examined using the Ct values of the baseline specimens and the baseline specimens that had been tampered with using DNA.

Instrument

The equipment used in this study included a Bio Safety Cabinet level 2, vortex, microtube, CR tube, filter tips, refrigerated centrifuge, micropipette, incubator, thermal cyclers (Tianlong Gentier 96 and BioRad CFX96), Nanophotometer NP80, Laminar Air Flow, marker, spindown, and computer. The materials used were the GeneAid Viral Nucleic Acid Extraction Kit, GoTaq Master Mix Kit Promega, GoTaq Probe Master Mix Kit Promega, Target Gene Primer S315G and Probe Set, 4% NaOH, 96-100% Ethanol, nuclease-free water, and hand gloves.

RESULTS

Analytical Specificity Results of the SYBR Green Method

The data obtained include 12 CT values, consisting of three CT values from the DNA baseline and nine CT values from triplicate measurements of the target DNA spiked with three different concentrations of interfering DNA. Table 1 shows that the lowest CT value obtained was 22.10, and the highest CT value was 23.65. The difference between the highest and lowest CT values for each variation did not exceed 1.50.

TABLE 1. CT Values for Analytical Specificity Determination of the SYBR Green Method

Composition Template	CT Value (3 replicates)			Mean
Sample Baseline	23.16	22.53	21.37	22.35
MTB: E. coli (5:1)	22.19	21.92	22.10	22.07
MTB: E. coli (5:3)	23.65	22.48	22.88	23.00
MTB: E. coli (1:1)	23.50	22.66	22.87	23.01

Because the data followed a normal distribution, a paired t-test was conducted to compare the average CT values of the baseline samples with those of each DNA interference concentration variation. The results shown in Table 2 had a 95% confidence level and a significance value of P > 0.05, indicating that there was no significant difference between the average CT values of the baseline specimens and those of the DNA interference concentration variations.

TABLE 2. Paired t-Test for the SYBR Green Method

Pair	Sig.
Baseline – MTB: E. coli (5:1)	0.693
Baseline – MTB: E. coli (5:3)	0.291
Baseline – MTB: E. coli (1:1)	0.263

Analytical Specificity Results of the TaqMan Probe Method

The data consisted of 12 CT values: three CT values for the target DNA and nine CT values for the target DNA spiked with three different concentrations of interfering DNA in triplicate. Table 3 shows that the CT values range from 19.36 to 20.24, with the difference between the highest and lowest CT values for each concentration variation not exceeding 1.00

TABLE 3. CT Values for Analytical Specificity Determination of the TaqMan Probe Method

Composition Template	CT Value (3 replicates)			Mean
Sample Baseline	19.93	19.57	19.86	19.79
MTB: E. coli (5:1)	19.75	19.58	20.24	19.86
MTB: E. coli (5:3)	19.86	20.05	19.48	19.80
MTB: E. coli (1:1)	19.36	19.77	19.90	19.68

The data distribution in Table 3 is normal, with a significance value of P > 0.05. The paired t-test results in Table 4 show significance values for each concentration variation pair above 0.650 and meet the P > 0.05 criterion, indicating that there was no significant difference in the average CT values between the baseline samples and spiked sample variations.

TABLE 4. Paired t-Test for the TaqMan Probe Method

Pair	Sig.
Baseline – MTB: E. coli (5:1)	0.712
Baseline – MTB: E. coli (5:3)	0.972
Baseline – MTB: E. coli (1:1)	0.685

Comparison of Analytical Specificity Between SYBR Green Method and TaqMan Probe Method

Based on Table 5, the significance value of the paired t-test average for the SYBR Green method was 0.398, while that the TaqMan Probe method was 0.790. This indicates that the analytical specificity of the TaqMan Probe method is 1.99 times greater than that of the SYBR Green method. This value indicates that a higher number reflects better specificity.

TABLE 5. Significance Values of the Paired t-Test for Both Methods

Pair	SYBR	TaqMan Probe
Baseline – MTB: E. coli (5:1)	0.693	0.712
Baseline – MTB: E. coli (5:3)	0.291	0.972
Baseline – MTB: E. coli (1:1)	0.263	0.685

DISCUSSION

This study aimed to compare the analytical specificity of *Mycobacterium tuberculosis* resistant to isoniazid detection using qPCR with the SYBR Green and TaqMan Probe methods. The research unit involved sputum, which was first extracted using the spin column method with a Viral Nucleic Acid Extraction Kit II from GeneAid. Additionally, the interfering DNA used in this study was pre-extracted. Broadly, four steps are involved in this method: cell lysis, nucleic acid binding, washing of interfering matrices, and nucleic acid elution (GeneAid, 2020). After the extraction, the concentration was measured using the drop method. The NanoDrop spectrophotometer used was a Nanophotometer NP80. The concentration of the target DNA obtained after measurement was 20.55 ng/µL, while the concentration of interfering DNA was 21.20 ng/µL.

For the SYBR Green method, the master mix used is Go Taq qPCR (Promega), which is used as the master mix. The master mix was prepared with a final concentration of 1X Go Taq qPCR, with

forward and reverse primers at 200 nM in a 20 µL reaction volume. A DNA template of 5 µL was then added. The PCR conditions were as follows: pre-denaturation phase of 1 cycle at 95°C for 2 min; denaturation phase of 40 cycles at 95°C for 15 s, and annealing extension phase of 40 cycles at 56°C for 1 min. These conditions were based on previous optimization studies.

For the TaqMan Probe method, the master mix used was the Go Taq Probe qPCR Promega. The master mix was prepared in a volume of 15 µL, containing a final concentration of 1X Go Taq Probe qPCR, forward and reverse primers at 400 nM, and probe at 300 nM. 2.5 µL of the DNA template was added. The PCR conditions for this method were as follows: pre-denaturation phase of 1 cycle at 95°C for 2 min; denaturation phase of 50 cycles at 95°C for 15 s; and annealing extension phase of 50 cycles at 62°C for 1 min.

To determine the analytical specificity for both SYBR Green and TaqMan Probe methods, the target DNA was spiked with interfering DNA in three variations. The ratios of target DNA to interfering DNA were 5:1, 5:3, and 1:1, resulting in interfering DNA concentrations of 17%, 38%, and 50%, respectively, in the template. Each concentration variation was repeated three times. The paired t-test results showed significance values of 0.398 and 0.790 for the SYBR Green method and TaqMan Probe method, respectively. In statistical terms, these significance values reflect the probability that the observed differences between paired data occur by chance. A higher significance value ($P > 0.05$) indicated that the differences between the groups were not statistically significant.

The SYBR Green method demonstrated a lower specificity than the TaqMan Probe, with a significance value of 0.398. Although statistically insignificant, this value suggests that the SYBR Green method may be less consistent in detecting differences between the samples. This is likely because SYBR Green is a dye that binds non-specifically to double-stranded DNA(Rodríguez-Lázaro & Hernández, 2013). It binds not only to the target PCR products but also to by-products such as primer dimers or nonspecific amplification products. This nonspecific binding can reduce the detection specificity and result in high background signals, diminishing the ability of the method to accurately differentiate between varying DNA concentrations. Since SYBR Green binds to all double-stranded DNA, the presence of nonspecific products can contribute to additional signals, making threshold cycle (Ct) analysis less precise (de Souza, 2019). Nonspecific products can interfere with the detection and measurement of the actual target DNA concentration. The efficiency of amplification with SYBR Green was influenced by primer quality and reaction conditions. If the primers are not fully specific or the reaction conditions are suboptimal, the results may be distorted, leading to less consistent Ct values.

The TaqMan Probe method is generally more robust in terms of amplification efficiency because specially designed probes enhance the amplification specificity. SYBR Green results often require additional analyses, such as melting curve analysis, to confirm the product specificity. If the melting curves are not properly analyzed, nonspecific products may not be detected, thereby reducing the specificity of the results(Baschien & Carl, 2020; Loftis & Reeves, 2012; Zhang et al., 2020). Thus, although SYBR Green is simpler and less expensive, its limitations in specificity and potential for nonspecific background signals can affect its consistency in detecting differences between samples, particularly in cases of low DNA concentrations or nonspecific amplification

products. SYBR Green remains a valuable method for various PCR applications, but its use requires attention to specificity and quality control. Using appropriate primer design and validation techniques, SYBR Green can provide valid and useful results in many situations (Meemetta et al., 2020; Rahmasari et al., 2022)

The TaqMan Probe method achieved a significance value of 0.790, indicating a higher specificity. This indicates that the TaqMan Probe method is better at distinguishing between concentration variations and provides more accurate results. The TaqMan Probe method uses specific probes that bind to the target DNA sequences and produce a fluorescent signal only when the probe is cleaved by DNA polymerase during amplification. This mechanism offers a more specific signal and reduces the risk of non-specific binding, thus enhancing detection accuracy and specificity. In contrast, SYBR Green lacks this specific mechanism, and the TaqMan Probe method employs probes designed to bind to specific DNA target sequences. These probes have unique sequences that bind only to specific targets, reducing the likelihood of non-specific amplification. This increases the detection specificity and helps to distinguish between DNA concentration variations more accurately. During amplification, the TaqMan Probe bound to the target DNA was cleaved by DNA polymerase, releasing a fluorescent signal.

This mechanism ensures that the fluorescent signal is highly specific and appears only when the probe is cleaved by the amplification of the target DNA. Thus, the fluorescent signal produced reflects only the amplification of the desired target, and not by-products or primer dimers, and minimizes the background signal from non-specific amplification products. Because a fluorescent signal is produced only when the probe is cleaved, the risk of non-specific amplification or contamination is reduced. Using specific probes, the TaqMan Probe provides more consistent results in detecting DNA concentration differences. This leads to more reliable data for distinguishing concentration variations, resulting in more accurate and reproducible results. Additionally, TaqMan Probes feature a quencher that absorbs the fluorescent signal before the probe is cleaved, preventing unwanted background signals and ensuring that only the signal from target amplification is measured.

According to the results, the analytical specificity of the TaqMan Probe method is 1.99 times greater than that of the SYBR Green method. This indicates that the TaqMan Probe is more effective in detecting differences in DNA concentrations, providing more reliable and accurate results. This enhanced specificity can be attributed to several factors. Specifically, the TaqMan probe uses probes designed to bind to unique target DNA sequences. These probes have a unique sequence that binds only to the specific target, reducing the likelihood of non-specific amplification or by-products that can cause unwanted signals. FRET Mechanism: The TaqMan Probe method utilizes Fluorescence Resonance Energy Transfer (FRET), involving two fluorescent molecules: one donor and one quencher. When the probe binds to the target and is cleaved during amplification, the donor releases a fluorescent signal.

FRET provides highly specific signals, as it occurs only when the probe is cleaved, minimizing background interference. The TaqMan probe produces a clearer and stronger fluorescent signal only when the target DNA is amplified. This improves the method's ability to accurately differentiate between variations in DNA concentration, as the signal is solely derived from the

amplification of the desired target and not from non-specific products. Reduced Primer Dimers: TaqMan Probe reduces the risk of primer dimers or non-specific amplification. This method detects only the target amplification product, thus minimizing false or inconsistent results that may occur with SYBR Green if primer dimers are formed. Improved Quality Control: TaqMan Probe offers better quality control because the fluorescent signal appears only after the probe is cleaved. This ensures that only signals from specific targets are detected, enhancing the accuracy and consistency of the results, which collectively contribute to the higher analytical specificity of the TaqMan Probe method compared to SYBR Green(Lou et al., 2022; Marinowic et al., 2021a; Tao et al., 2022; Ugwu et al., 2023).

Although none of the methods showed statistically significant differences in paired t-test results, the TaqMan Probe method demonstrated better analytical specificity than SYBR Green. This is crucial for applications that require highly accurate and consistent detection. Therefore, the TaqMan Probe may be more advisable in situations in which specificity is a key factor. However, SYBR Green remains useful for various PCR applications, but requires careful attention to specificity and quality control. The evaluation of SYBR Green for detecting SARS-CoV-2 showed that it performs similarly to TaqMan-based methods, thus offering a viable alternative for diagnostic testing. Additionally, SYBR Green can be used to detect herpes virus (Dorlass et al., 2020; Marinowic et al., 2021b; Meemetta et al., 2020; Pereira-Gómez et al., 2021; Rahmasari et al., 2022)

CONCLUSION

Based on the study results, it can be concluded that the analytical specificity of the TaqMan Probe method for detecting Mycobacterium tuberculosis resistant to isoniazid using qPCR was higher than that of the SYBR Green method. However, both these methods can be used for diagnostic purposes.

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CONFLICT OF INTEREST

No conflict of interest

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