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Optimization of Annealing Temperature and Primer Concentration for Detection of Mycobacterium Tuberculosis Isoniazid Resistance Real-Time PCR Method

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ABSTRACT

Indonesia is ranked second in the number of TB cases in the world, according to the WHO report for 2022. The National TB Eradication Program is difficult to carry out because of the presence of Mycobacterium tuberculosis (M. tb) bacteria, which are resistant to various anti-tuberculosis drugs (OAT), known as Multidrug Resistant Tuberculosis (MDR-TB). The drug is resistant to two anti-tuberculosis drugs (OAT), namely isoniazid (INH) and rifampicin. The development of a nucleic acid-based diagnostic kit designed to directly detect resistance to INH with mutations in the KatG gene codon 315 (S315T, S315N, S315I, S315R, and S315G), codon R463L, and sensitive M. tb. rifampicin using the real-time PCR method. The PCR components carried out in the research include annealing temperature and primer concentration. **Objective:** This research aims to determine the annealing temperature and optimum primer concentration. **Methods:** Quasi Experiment. The data obtained is primary data obtained from the results of the amplification curve in the form of Ct (cycle threshold) values from each reaction treated with variations in annealing temperature and variations in primer concentration.**Results:** The optimal annealing temperature for detecting isoniazid-resistant Mycobacterium tuberculosis using real-time PCR on the KatG gene mutation codons S315T, S315N, and S315G is 55 oC. Meanwhile, the S315I, S315R, and R463L codons are 56 oC. And the primary Mycobacterium tuberculosis Rifampicin sensitivity is 60 oC. The optimal primer concentration for the KatG gene mutation codons S315T, S315I, S315G, R463L, and Mycobacterium tuberculosis Rifampicin-sensitive is 400 nM. Meanwhile, the S315N codon is 200 nM. Meanwhile, on the S315G, it is 300 nM. **Conclusion:** The conclusion of this research is that the annealing temperature is 55–560 °C and the optimal primer concentration is in the range of 200–300 nM.

KEYWORDS: Primary Concentration | Annealing Temperature | *Mycobacterium tuberculosis* | Isoniazid Resistance| Real Time PCR

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I. INTRODUCTION

Tuberculosis or TB is a disease caused by infection with the bacteria Mycobacterium tuberculosis (M. tb) in the lungs [1]. According to the 2022 WHO Report, Indonesia is ranked second in terms of TB cases with 969,000 cases accounting for 9.2% of global TB cases [2].

The diagnosis of a patient with INH monoresistance can be known after the patient has completed treatment for 6 months, but after microscopic examination it still shows the presence of M. TB bacteria, so further examination is carried out by carrying out culture and TCM XDR (extensive drug resistance) examination[3]. This causes the treatment of patients who are INH-resistant to be very long . Therefore, currently a nucleic acid-based diagnostic kit is being developed that can directly detect resistance to INH using the Real-Time PCR (qPCR) method. The qPCR method is a fast, sensitive, and specific method compared to culture for checking resistance [4]. This method can also amplify several different genes using various specific primers in one reaction (qPCR Multiplex). That is hoped

that it can speed up the process of diagnosing drug resistance cases in one process [5].

This diagnostic kit is designed to detect mutations in the M. tb katG gene codons 315 and 463 [6]. Research on primer design that detects mutations in these two codons produces 6 pairs of primers, namely the amino acid serine (S) to threonine (S315T), asparagine (S315N), isoleucine (S315I), arginine (S315R), or glycine (S315G) and mutation of the amino acid arginine to leucine (R463L). However, in vitro testing has not been carried out in this study [5]. An optimization process is necessary for newly designed diagnostic kits because they must be used under optimal circumstances to detect the desired target gene. Important optimization processes include annealing temperature and primer concentration [6]. Setting the annealing temperature is very important for optimization because the annealing stage determines how optimally the primer attaches to the DNA template, while the primer concentration used will also affect the effectiveness of the PCR reaction [7].

The annealing temperature used must be adjusted to the melting temperature (Tm) or melting temperature of the primer [7–10]. Choosing an annealing temperature that is too low will cause non-specific attachment, while a temperature that is too high can make it difficult to form primer and DNA template bonds [11]. In general, the annealing temperature range is 36°C to 72°C with a time of 30-45 seconds[12]. Optimizing the annealing temperature of a primer can be done by reducing the Tm value by 5°C [13]. Using primer concentrations that are too high results in PCR products that are increasingly non-specific. [14].

The success of the qPCR process is proven by the formation of an amplification curve and providing a cycle threshold (Ct) value [15]. It is the number of cycles in which the reaction fluorescence signal crosses the threshold. Based on this background, the author wants to research "Optimization of Annealing Temperature and Primer Concentration for Detection of Isoniazid-Resistant Mycobacterium tuberculosis using the Real-Time PCR Method ".

II. METHODS

The research used a Quasi Experiment with variations in the optimization of annealing temperature and primer concentration for the detection of Mycobacterium tuberculosis katG gene mutations in codon 315 (S315T, S315N, S315I, S315R, S315G) and codon 463 (R463L) using the Real-Time PCR method. The sample population was sputum samples confirmed positive for isoniazid-resistant Mycobacterium tuberculosis obtained from the West Java Provincial Health Laboratory.The patient who was confirmed positive for Mycobacterium tuberculosis was sensitive to Rifampicin from the West Bandung Regency Health Lab. The temperature variations used range from 52-650C depending on the Tm of each primer used. The data obtained is primary data obtained from the results of the amplification curve in the form of Ct (*Cycle threshold*) values from each reaction treated with variations in annealing temperature and variations in primer concentration. The optimality of the reaction is determined by observing the CT value resulting from the reaction in each treatment. The treatment with the lowest CT value at a certain primer concentration and annealing temperature is determined as the optimal result.

Spin Column method DNA Extraction (Geneaid, 2020)

A 200 hundred µl mucopurulent/viscous greenish yellow phlegm pattern turned into placed right into a 1.5 mL effendorf/microtube tube, then diluted with 4% NaOH answer with a ratio of 1:1. The pattern mixture turned into centrifuged at a speed of 15,000 rpm for two minutes, then the supernatant changed into discarded. Then upload 400 µl VB lysis buffer to the tube and vortex then incubate for 10 mins. Then upload 450µL ad buffer and vortex. After that, the VB Column is placed on the pinnacle of the gathering tube and then transfer six hundred µL of the lysate combination into the VB Column. The tube was centrifuged at 15,000 rpm for 1 minute. Then discard the liquid in the collection Tube replace the VB Column and position the final lysate again into the VB Column and centrifuge at 15,000 rpm for 1 minute. Then add four hundred µL W1 Buffer into the VB Column then centrifuge at 15,000 rpm for 30 seconds. Then add six hundredµL Wash Buffer to the VB Column then centrifuge at 15,000rpm for 3 mins 30 seconds. The VB Column is transferred to the Microcentrifuge Tube. Then 50 µL of RNAse-loose water was delivered into the VB Column.

Optimization of Annealing Temperature and Primer Concentration

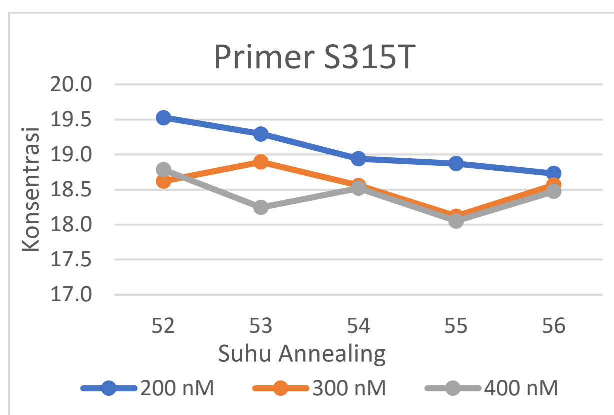
The PCR settings used in this examination have been 1 initial denaturation cycle of 10 mins. Pre-Denaturation for two mins, Denaturation for 15 seconds, annealing for 1 minute, and extension for 1 minute. Samples with various concentrations of each primer (200nM; 300nM; 400nM) at annealing temperatures that correspond to the Tm of each primer. The statistics that have been acquired are analyzed with the use of an amplification curve. The optimality of the reaction is determined by way of gazing at the Ct cost as a result of the reaction on every pattern.

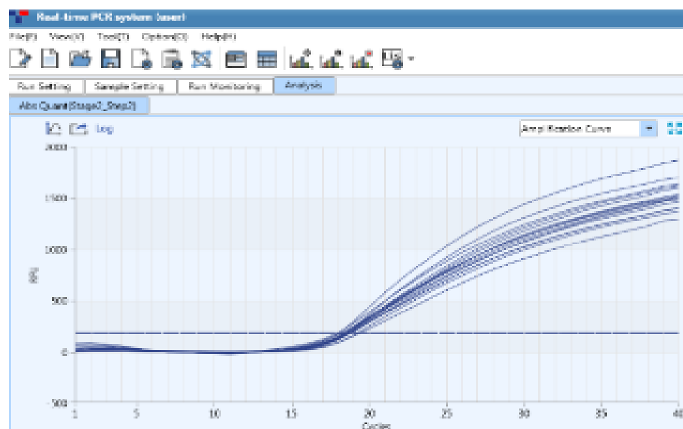
III. INTERPRETATION OF RESULTS

Optimization of annealing temperature on primer S315T

PCR on the goal gene primer S315T with an annealing temperature of fifty two-fifty six°C fashioned a curve indicating the amplification of isoniazid-resistant Mycobacterium tuberculosis the usage of an ahead primer and an opposite primer that had a Tm of fifty nine°C. For the S315T goal gene with a primer awareness of 200 nM, the lowest CT fee was obtained at an annealing temperature of fifty six°C. At an awareness of 300 nM, the lowest CT cost was acquired at an annealing temperature of fifty five°C. in the meantime, at a attention of four hundred nM, the lowest CT cost is received at an annealing temperature of 55°C.

Optimization of Annealing Temperature and Primer Concentration for



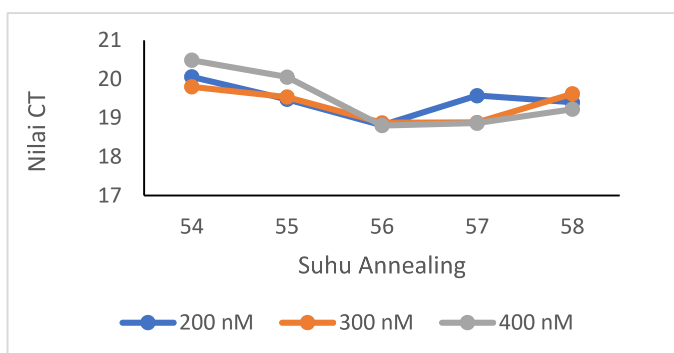


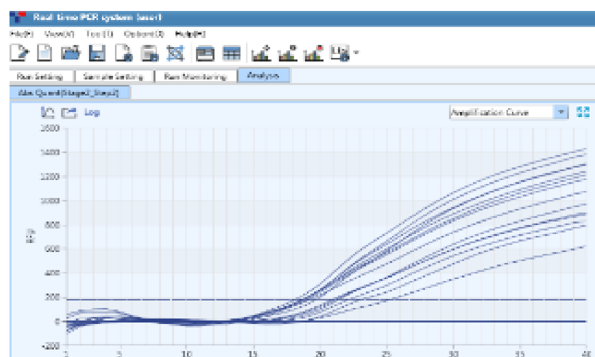
Picture 1 PCR consequences of S315N

Optimization of annealing temperature on primer S315I

The primer for the S315I target gene with an annealing temperature of 54-58°C formed a curve indicating the amplification of isoniazid-resistant Mycobacterium tuberculosis using a forward primer and a reverse primer that had a Tm of 61°C.

on the target gene S315I with a concentration of 200 nM, the lowest CT value was obtained at an annealing temperature of 56°C. At a concentration of 300 nM, the lowest CT value was obtained at an annealing temperature of 56°C. Meanwhile, at a concentration of 400 nM, the lowest CT value was obtained at an annealing temperature of 56°C.



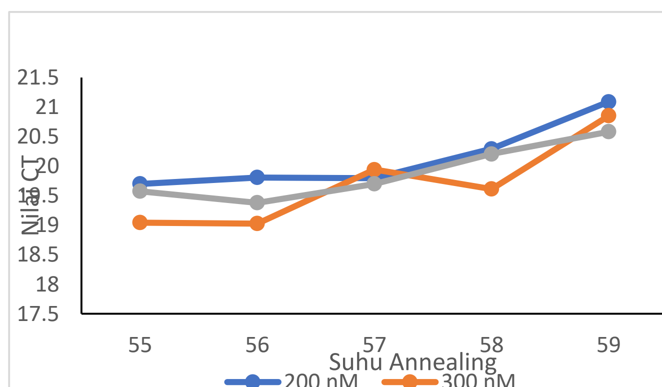


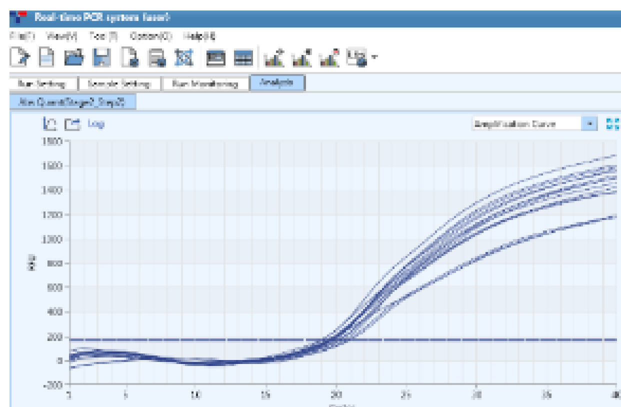
Picture 3 Primary PCR Results of S315I

Optimization of annealing temperature on primer S315R

On the S315R primer with an annealing temperature of 55-59°C, a curve was formed indicating the amplification of isoniazid-resistant Mycobacterium tuberculosis using the forward primer and reverse primer which had a Tm of 62°C.

For the S315R target gene with a concentration of 200 nM, the lowest CT value was obtained at an annealing temperature of 55°C. At a concentration of 300 nM, the lowest CT value was obtained at an annealing temperature of 56°C. Meanwhile, at a concentration of 400 nM, the lowest CT value was obtained at an annealing temperature of 56°C.





Picture 4 Primary PCR Results of S315R

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Optimization of annealing temperature on primer S315G

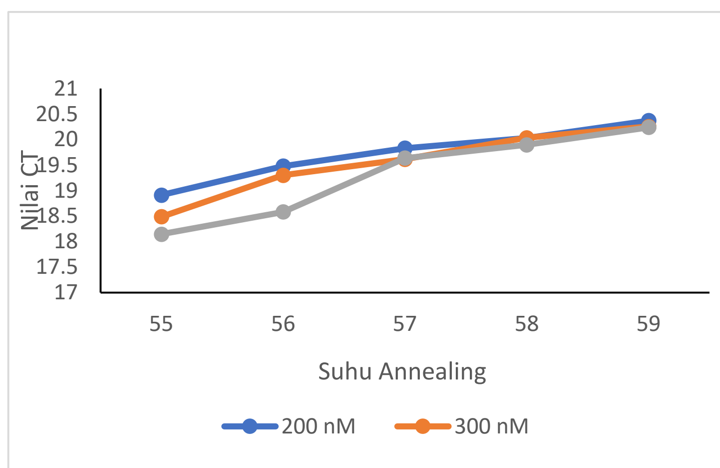
On the S315G primer with an annealing temperature of 55-59°C, a curve was formed indicating the amplification of isoniazid-resistant Mycobacterium tuberculosis using the forward primer and reverse primer which had a Tm of 62°C.

For the target gene S315G with a concentration of 200 nM, the lowest CT value was obtained at an annealing temperature of 55°C. At a concentration of 300 nM, the lowest CT value was obtained at an annealing temperature of 55°C. Meanwhile, at a concentration of 400 nM, the lowest CT value was obtained at an annealing temperature of 55°C.

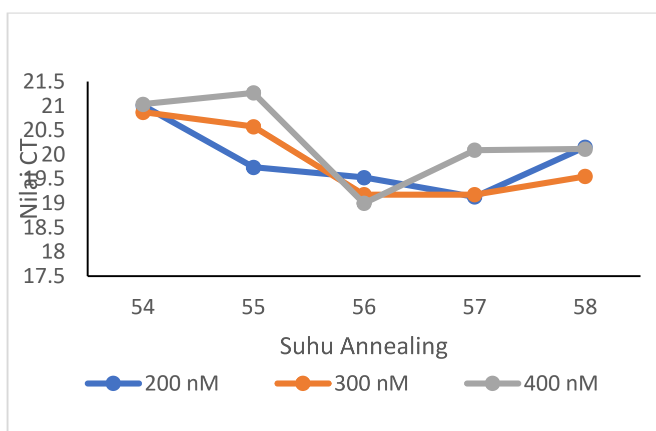
Optimization of annealing temperature on primer R463L

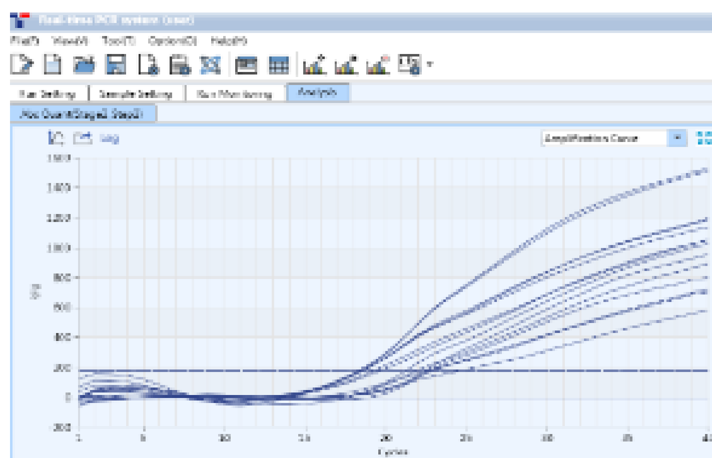
In the R463L primer using varying annealing temperatures from 54-58°C, a curve was formed indicating the amplification of isoniazid-resistant Mycobacterium tuberculosis using the forward primer and reverse primer which had a Tm of 61°C.

On the target gene R463L with a concentration of 200 nM, the lowest CT value was obtained at an annealing temperature of 57°C. At a concentration of 300nM, the lowest CT value was obtained at an annealing temperature of 56°C. Meanwhile, at a concentration of 400 nM, the lowest CT value was obtained at an annealing temperature of 56°C.



Picture 5. Primary PCR Results of S315G





Picture 6 Primary PCR Results of R463L

Optimization of annealing temperature on primer *Mycobacterium tuberculosis* Rifampicin Sensitif

In the M.Tb Rifampicin Sensitive primer using a variation of annealing temperature from 52-56°C, a curve was formed indicating the amplification of isoniazid-resistant Mycobacterium tuberculosis using the forward primer and reverse primer which had a Tm of 59°C.

For the M.TB target gene Rifampicin sensitive with a concentration of 200 nM, the lowest CT value was obtained at an annealing temperature of 56°C. At a concentration of 300 nM, the lowest CT value was obtained at an annealing temperature of 55°C. Meanwhile, at a concentration of 400 nM, the lowest CT value was obtained at an annealing temperature of 55°C.

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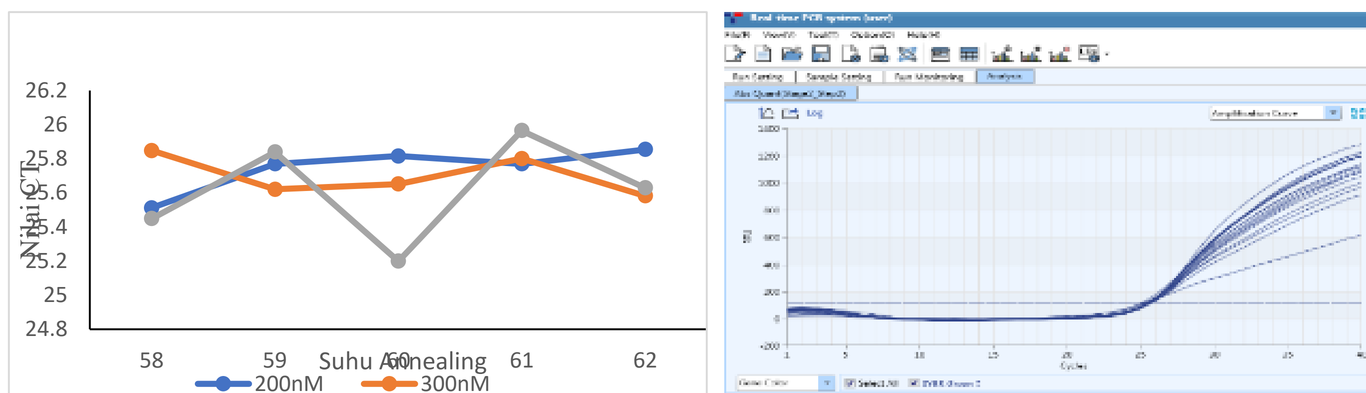

Picture 7 Primary PCR Results of M. Tb Rifampicin Sensitif

Data analysis

Annealing temperature 55 oC with a concentration of 400 nM. on the S315N primer, the lowest CT value was obtained at 18,699 at an annealing temperature of 55oC with a concentration of 200 nM. In the S315I primer, there was an increase in the CT value at the annealing temperature of 56oC with a concentration of 400 nM. In the S315R primer, there was an increase in the CT value at the annealing temperature of 56oC with a concentration of 300 nM. In the S315G primer, there is an increase in the CT value at an annealing temperature of 55oC with a concentration of 400 nM. In the R463L primer, there was an increase in the CT value at the annealing temperature of 56oC with a concentration of 400 nM. And in the Rifampicin Sensitive Mycobacterium tuberculosis primer, there was an increase in the CT value at an annealing temperature of 60oC with a concentration of 400 nM.

IV. RESULTS AND DISCUSSION

Annealing temperature 55^{0}C with an attention of 400 nM. on the S315N primer, the lowest CT cost turned into obtained at 18,699 at an annealing temperature of 55°C with an awareness of 200 nM. within the S315I primer there was an increase inside the CT cost at the annealing temperature of 56°C with an attention of 400 nM. inside the S315R primer, there was a growth inside the CT fee on the annealing temperature of 56°C with an attention of three hundred nM. within the S315G primer, there may be an increase inside the CT price at an annealing temperature of 55°C with an attention of four hundred nM. in the R463L primer there has been a growth inside the CT price at the annealing temperature of 56°C with an attention of four hundred nM. And inside the Rifampicin sensitive Mycobacterium tuberculosis primer there was a boom within the CT cost at an annealing temperature of 60oC with an attention of 400 nM. The one of the factors that influences the success of the Real-Time PCR process is the annealing temperature. Determining the annealing temperature used can be calculated based on (Tm-5) °C to (Tm +5) °C [13]. Tm (melting temperature) is the temperature at which half of the double DNA strands separate. The Tm value will affect the denaturation temperature of the DNA double helix strand and the primer annealing temperature. Primers with a Tm that is too high, exceeding 70°C, will easily experience mispriming at low temperatures. Apart from that, the formation of a bond that is too strong between the DNA template and the primer will result in a low PCR product [10]. Meanwhile, primers with a low Tm will not be able to work at high temperatures. The Tm of the designed primer meets the criteria, namely in the range of 59-62°C. The primary Tm design results have met the requirements for a good primer [11].

Apart from the melting temperature, the percent (%) GC factor has an influence on the bonds between DNA strands. The amount of guanine and cytosine in a primer, the GC% should be in the range of 40-60% [16] . The designed primers have a GC% of 52 – 54% which is still within the GC% criteria range, except for the S315N primer which has a GC% of 67%. Primers with a low % GC can reduce the efficiency of the PCR process because the primers are unable to compete to attach effectively to the template [13] . A high GC percentage will result in strong bonds between DNA strands because GC contains more bonds between nucleotides than AT so it will affect the Tm value [17]. Besides optimization on annealing temperature, primer concentration is very important too. The primer functions as a barrier for the target DNA fragment to be amplified [13]. High concentrations will result in sticking errors so that undesirable products can be synthesized [18]. Adding a primer concentration that is too low can cause no amplification so the PCR result becomes a false negative [13]. Increasing the primer concentration implies that more primers are attached to the annealing process, which can accelerate the intersection of the amplification curve [19] . The higher the primary concentration, the lower the Ct value.

From the research results, it was found that the lowest Ct value at the optimal concentration of 400 nM occurred for all primers except for primers S315N and S315R. The results of the research obtained different concentrations, namely 200nM for the S315N primer and 300 nM for the S315R primer. This is not by the theory that the greater the primary concentration used, the smaller the Ct value obtained [16]. This can happen due to several factors, the cause is due to the dissolving technique during mixing, less precise pipetting of the DNA template or the influence of the PCR equipment used on whether the heating block is in optimal condition or not [20]. Errors such as reagent dissolution techniques or errors when pipetting the DNA template volume cause the concentration of the primer or DNA template to decrease so that the CT value increases [21].

Through this research, the optimal annealing temperature for the detection of isoniazid-resistant Mycobacterum tuberculosis was found to be 55-56^{0}C. At this temperature, the sample with a concentration of 400nM shows the lowest Ct value.In research conducted by Heck

CONCLUSIONS

The annealing temperature with the lowest CT value for detecting isoniazid- resistant Mycobacterium tuberculosis using Real-Time PCR on the KatG gene mutation codons S315T, S315N, and S315G is 55ºC. Meanwhile, the S315I, S315R and R463L codons are 56ºC. And the primary Mycobacterium tuberculosis Rifampicin sensitivity is 60ºC. However, from all the CT value results there were no significant differences between all the temperature variations used, so it can be said that the annealing temperature of 52-60 ºC is the optimal temperature and can be used to examine isoniazid resistance Mycobacterium tuberculosis.

The primer concentration with the lowest CT value for detecting isoniazid-resistant Mycobacterium tuberculosis using Real-Time PCR on mutations in the KatG codon S315T, S315I, S315G, R463L, and Mycobacterium tuberculosis Rifampicin sensitive gene is 400nM. Meanwhile, for the S315N codon, the lowest CT value is 200nM. And the lowest CT value on S315G is 300nM. The CT value obtained does not have much difference and all products can be amplified so that all variations in primer concentration can amplify the product. The results show that there is no significant difference between all the concentration variations used and it is still within the optimum CT value limit, so it can be said that the concentration 200-400 nM is the optimal concentration and can be used to screen for isoniazid resistance Mycobacterium tuberculosis.

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