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Optimization of the boil loop-mediated isothermal amplification (LAMP) method for detection of Mycobacterium tuberculosis in saliva

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Abstract: Indonesia has the second-highest tuberculosis (TB) burden worldwide. Conventional TB diagnostic methods in Indonesia are limited by sensitivity, processing time, and cost. The Boil Loop-Mediated Isothermal Amplification (Boil LAMP) method using saliva offers a potential alternative. This study aimed to determine the optimal conditions of the Boil LAMP method for detecting the *gyrB* gene of *Mycobacterium tuberculosis* in saliva. Saliva samples were collected from 15 TB patients in Cimahi, Indonesia. Genomic DNA was extracted using simple and modified boiling methods. Optimization of the LAMP assay was performed using genomic DNA concentrations of 25, 50, and 100 ng/µl and amplification temperatures of 59–62°C. Amplification products were analyzed by agarose gel electrophoresis. The modified boiling method yielded salivary genomic DNA with an average concentration of 139 ng/µl and a purity of 1.8. The 320 bp *gyrB* gene was successfully amplified, with the strongest amplification observed at a DNA concentration of 100 ng/µl and a temperature of 61°C. In conclusion, the modified boiling method combined with the LAMP assay using 100 ng/µl genomic DNA at 61°C provides optimal conditions for detecting *M. tuberculosis* in saliva, offering a rapid, non-invasive, and cost-effective diagnostic approach for TB.

Keywords: Tuberculosis; Boil LAMP; Mycobacterium tuberculosis; Saliva; Molecular diagnosis.

INTRODUCTION

Tuberculosis (TB) is an infectious disease caused by *Mycobacterium tuberculosis*[1]. According to the World Health Organization, TB is the infectious disease with the highest global mortality rate[2]. In 2021, the world's number of TB cases reached 10.6 million. Indonesia placed second among the eight countries with the highest number of TB cases, with nearly 1 million cases (9.2% of the world TB burden)[3].

The World Health Organization has specified three methods for TB testing: bacterial culture, microscopic examination of sputum, and molecular rapid tests[4]. Bacterial culture examination is the gold standard for TB testing, but it takes 2–8 weeks[5,6]. Sputum microscopic examination with Ziehl-Neelsen stain is commonly used as the basis for TB diagnosis and control, but it has a low sensitivity (20–80%)[5]. It takes a large number of bacteria (10^4–10^5 bacteria per ml) to get a positive result on the test[7]. Molecular rapid tests are more sensitive than the previous two

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methods, but they require expensive specialized equipment. Because of the short amplification process, rapid molecular tests could generate false negative results[8].

This is important to developing another method for TB screening, namely Loop-Mediated Isothermal Amplification (LAMP)[9–13]. LAMP is a DNA amplification technique that uses 2-3 pairs of primers at a constant temperature of 60–65°C[9,14]. This method has a sensitivity value of more than 87% and a specificity value of more than 94%[9,10,15]. LAMP does not require expensive tools such as Polymerase Chain Reaction (PCR), making it more suitable for use in developing countries with a high number of TB cases[9].

The LAMP method requires a sample of genomic DNA to test for TB. A DNA extraction kit is generally used to extract genomic DNA[11–13]. Despite the high concentration and purity of genomic DNA extracted with the kit, the kit is costly and the processing time is lengthy[16]. The boiling method, on the other hand, is a simpler, cheaper, and faster method of extracting DNA[17,18]. This method only uses a high temperature to lyse cells. Several studies reported that boiling extraction and LAMP (Boil LAMP) methods were effective for detecting TB[19,20].

The specimen used to detect TB by the Boil LAMP method is sputum[19,20]. Sputum collection is difficult for some patients, especially children, and can endanger health workers. Sputum quality is also highly influenced by the patient's understanding of proper sputum collection techniques[21]. Saliva is another specimen that can be used to test for TB.

The use of saliva in TB examination can minimize pre-analytic errors when collecting specimens. Saliva is a non-invasive sample with numerous clinical applications. The previous study has shown that *M. tuberculosis* can be detected in saliva with a high level of accuracy[22]. The results of other studies also showed that *M. tuberculosis* detection in saliva was 92% accurate[23]. However, some of these studies still use the PCR method to detect TB in saliva, not using the Boil LAMP method. Therefore, this present study aims to determine the optimal conditions of Boil LAMP method to detect *M. tuberculosis* in saliva.

MATERIAL AND METHOD

This study was conducted between March and June 2022 at the Laboratory of Molecular Biology, Faculty of Health Sciences and Technology, Jenderal Achmad Yani University. The research method used was experimental. The optimal conditions for amplification of the gyrB gene from *M. tuberculosis* by the Boil LAMP method in saliva were determined using various concentrations of genomic DNA and amplification temperature.

The sampling technique used is quota sampling. Saliva samples were collected from 15 TB patients who had repeated treatment at various health centers in Cimahi. Previous treatment failures in the study sample were due to irregularity in taking medication. In this study participants were TB patients undergoing retreatment due to previous non-adherence/failed treatment. Saliva was collected to evaluate the feasibility of *gyrB* amplification using Boil LAMP. These participants were informed about the study's background, objectives, and procedures, and they signed the informed consent form. The entire study procedure was also approved by the Health Research Ethics Commission, Faculty of Health Science and Technology, Jenderal Achmad Yani University under ethical approval number 11/KEPK/FITKES-UNJANI/IV/2022.

This study commenced by comparing the results of salivary genomic DNA extraction using a simple and modified boiling method. In the simple boiling method, 200 µl of saliva was heated at 95°C for 15 minutes. The saliva was then

centrifuged at 12,000 rpm for 10 minutes. The formed pellet was resuspended in 50 µl of TE buffer. In the modified boiling method, 200 µl of saliva was heated at 95°C for 20 minutes. After that, the saliva was homogenized with 200 µl of chloroform, 300 µl of isopropanol, and 1 ml of 70% ethanol. The suspension was centrifuged at 12,000 rpm for 5 minutes. The formed pellet was resuspended in 200 µl of TE buffer. In this study, sputum from TB patients was represented as the positive control. Sputum genomic DNA was extracted using the same method, but it was first decontaminated with 4% NaOH and sterile phosphate buffer (pH 6.8). The extracted genomic DNA was visualized using 1% agarose gel electrophoresis. The concentration and purity of the extracted genomic DNA were determined using spectrophotometric methods.

Table 1. Primer specifications

Primer Type	Primer Name	DNA Sequence
Inner	FIP	AGACCACTCGTACCCGTCGCCGGTGGTTAACGCGCTAT
	BIP	ATGAGAAGTCGGAACCCCTGGGACCGTTGACCCCGTCTTC
Outer	F3	GCGATATCTGGTGGTCTG
	B3	CCGTGGTTTCGAAAACAGC
Loop	Loop F	AACTAGAGCTGAAGCTCGG
	Loop B	CCTCAAGCAAGGGGCG

The gyrB gene was amplified using the LAMP method after the genomic DNA was successfully extracted. The primer sequence used in this study was obtained from the Standard Operational Guidelines for the LAMP Molecular Diagnostic Test for Rapid Detection of Pulmonary TB in Indonesia[24] (Table 1), but the reaction composition and amplification conditions were modified. The amplification reaction had a total volume of 25 µl which included 2.5 µl of *Bsm* DNA Polymerase Buffer (Thermo Fisher Scientific) 10X; 4 µl of $MgCl_2$ (Thermo Scientific) 5 mM; 4 µl of betaine (Sigma-Aldrich) 5 M; 3.5 µl of dNTP mix (Thermo Fisher Scientific) 10 mM; 1 µl of inner primer 40 µM; 0.5 µl outer primer 10 µM; 1 µl of loop primer 10 µM; 6.5 µl of nuclease-free water; 1 µl of *Bsm* DNA Polymerase (Thermo Fisher Scientific) 8U/µl; and 1 µl genomic DNA. The genomic DNA concentrations used were 25 ng/µl, 50 ng/µl, and 100 ng/µl. The solution was incubated at temperatures ranging from 59 to 62°C for 1 hour. The amplification reaction was inactivated for 2 minutes at 80°C. Amplicons were visualized by the 2% agarose gel electrophoresis method. After obtaining the optimum conditions, the gyrB gene was detected in 15 research samples.

RESULTS AND DISCUSSION

The optimization of the Boil LAMP method to detect the gyrB gene in saliva has been successfully completed. The method started with the extraction of salivary genomic DNA (Figure 1). Only lanes 2, 5, 6, and 7 on the electropherogram had DNA bands larger than 10,000 bp. The results demonstrated that salivary genomic DNA could be extracted successfully using the modified boiling method but not the simple boiling method. According to the spectrophotometric method results (λ=260/280), the average concentration of salivary genomic DNA extracted was 139 ng/µl with a purity value of 1.8.

After determining the optimum conditions of genomic DNA extraction, different concentrations of genomic DNA and amplification temperatures were selected to optimize gyrB gene amplification (Figure 2). The optimization of *gyrB* gene amplification was performed using different genomic DNA concentrations and amplification temperatures (Figure 2). Amplification using a genomic DNA concentration of 25 ng/µl produced faint DNA bands and was observed only at amplification temperatures of 60–62°C (Figure 2A, lanes 3–5).

Amplification using 50 ng/µl genomic DNA generated clearer DNA bands across amplification temperatures of 59–62°C (Figure 2B, lanes 2–5).

The thickest DNA bands were obtained using a genomic DNA concentration of 100 ng/µl across amplification temperatures of 59–62°C, with the strongest amplification observed at 61°C (Figure 2C, lane 4).

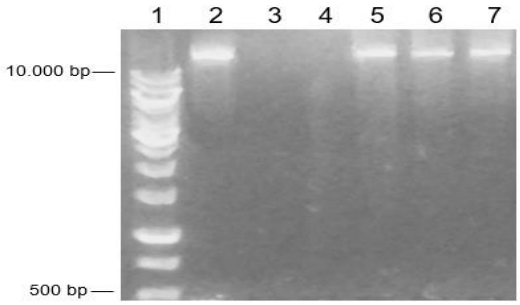

Figure 1. Electrophoresis Results of Saliva Genomic DNA Extraction.

Note: Lane 1: Thermo Scientific DNA Ladder 1 kb; Lane 2: Positive Control (Sputum); Lane 3: Negative Control (ddH_2O); Lane 4: Simple Boiling Water Method; Lane 5-7: Modified Boiling Methods.

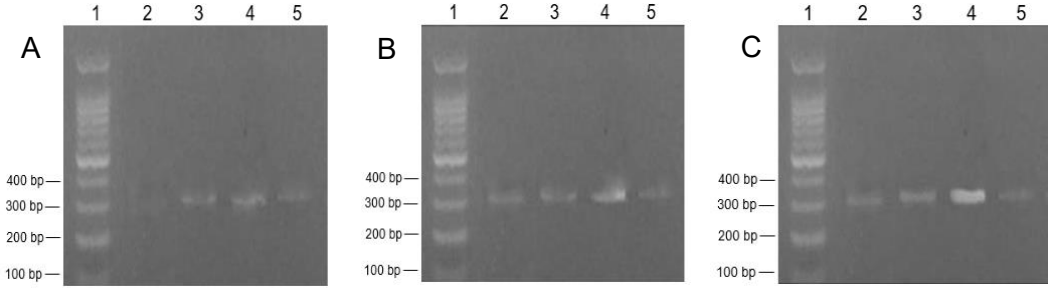

Figure 2. Electrophoresis Results of the Optimization Result of Genomic DNA Concentration and Amplification Temperature for gyrB Gene Using the LAMP Method.

Note: A: 25 ng/µl Genomic DNA concentration; B: 50 ng/µl Genomic DNA concentration; C: 100 ng/µl Genomic DNA concentration. Lane 1: Thermo scientific DNA Ladder (100 bp); Lane 2: Amplification Temperature at 59°C; Lane 3: Amplification Temperature at 60°C; Lane 4: Amplification Temperature at 61°C; Lane 5: Amplification Temperature at 62°C

The best amplification conditions were then used to detect the gyrB gene in 15 research samples. All research samples had 320 bp DNA bands on the electropherogram (Figure 3). This indicates that the gyrB gene found in the saliva samples was amplified successfully. The size of the DNA band was similar to that of the positive control. The absence of DNA bands formed in negative controls such as ddH_2O, sputum, and saliva from healthy people indicated that the amplification process occurred specifically.

This study indicated that the simple boiling method could not be used to extract salivary genomic DNA. The simple boiling method involves heating at high temperatures to increase the permeability of the cell wall, damaging the structure of the cell wall and membrane, allowing DNA to exit the cell[25,26]. This method only includes a cell lysis step, whereas prior study has shown that DNA extraction from saliva requires cell lysis, precipitation, and DNA purification[27].

The modified boiling method was used successfully to extract salivary genomic DNA. The concentration and purity of the DNA were particularly good. The optimal extracted DNA concentration was 5–100 ng/µl, and good DNA purity was 1.8–2.0[28]. The addition of reagents to the boiling method, such as chloroform, isopropanol, and 70% ethanol, has been shown to optimize the process of extracting sputum genomic DNA[19,29]. Chloroform removes cell debris and can

denature proteins and polysaccharides[28]. Heating and adding chloroform helps inactivate bacteria and denature bacterial cell walls which contain thick fat[29]. Isopropanol buffer helps in the DNA precipitation process, while 70% ethanol supports the DNA purification step[30].

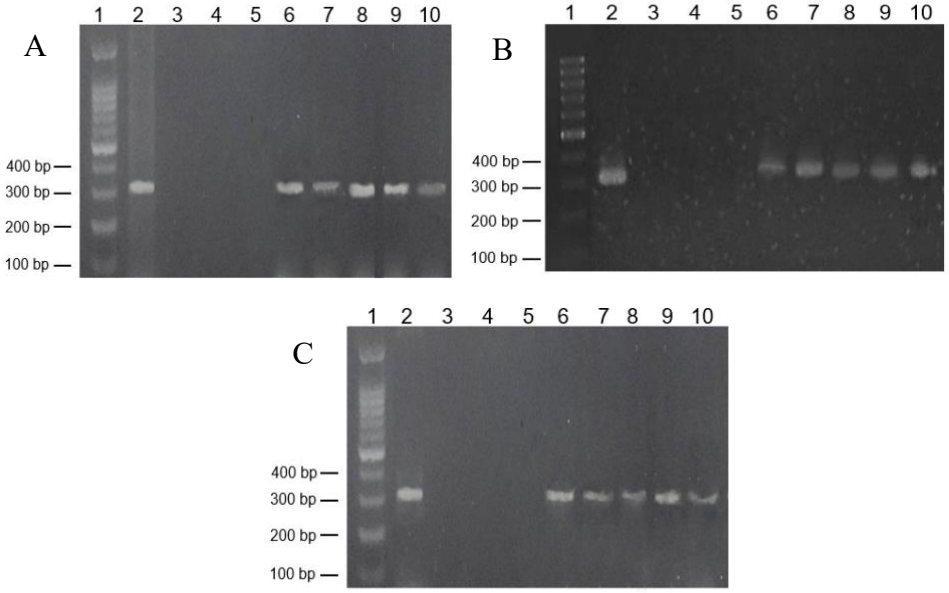

Figure 3. Electrophoresis Results of the gyrB gene amplification results in the sample.

Note: Lane 1: Thermo scientific DNA Ladder (100 bp); Lane 2: Positive Control (Sputum); Lane 3: Negative Control (ddH_2O); Lane 4: Negative Control (Healthy People's Sputum); Lane 5: Negative Control (Healthy People's Saliva); Lane 6-10: (A) Sample Numbers 1-5; (B) Sample Numbers 6-10; (C) Sample Numbers 11-15.

Furthermore, this study showed that the Boil LAMP method was successfully used to amplify the gyrB gene from saliva specimens. The gyrB gene is a conserved gene in *M. tuberculosis* that encodes the topoisomerase enzyme[31]. Therefore, gyrB has the potential to be used as a TB marker gene. The 320 bp gene can be amplified with three pairs of primers: inner, outer, and loop primers[24,32]. The most commonly used inner, outer, and loop primer concentration ratios are 1:2:8[16]. Previous research has shown that the primer sequence used in this study can specifically identify the gyrB gene[32].

Aside from primers, the gyrB gene amplification procedure involves Bsm DNA Polymerase buffer, $MgCl_2$, betaine, and dNTP mix. The Bsm DNA Polymerase buffer contains salt to maintain pH stability, and tween 20 to increase detection sensitivity[13]. Mg^{2+} ions derived from $MgSO_4$ (contained in Bsm DNA Polymerase buffer) and $MgCl_2$ function as cofactors for DNA polymerase enzymes. Betaine is a zwitterionic amino acid which has the ability to reduce the secondary structure of G-C DNA sequences and inhibit non-specific amplification in the LAMP reaction[33]. The dNTP mix contains nitrogenous bases, which compose the DNA strand.

The optimization results showed that a genomic DNA concentration of 100 ng/µl produced a thicker DNA amplicon band than 25 ng/µl and 50 ng/ µl. Because genomic DNA provides as a template during the amplification, it is an important factor in determining the quality of the DNA amplicon band. A low concentration of DNA resulted in fewer amplicons, but a high concentration of DNA could also inhibit the amplification reaction[16]. The inhibitor content of the polymerization reaction increases with the higher DNA concentration used.

Furthermore, the optimization results showed that the optimal amplification temperature was 61°C. The amplification temperature affects the activity of the

polymerase enzyme used, Bsm DNA Polymerase. The enzyme is derived from the thermophilic bacterium *Bacillus smiithii* and can catalyze the synthesis of DNA strands in the 5'→3' direction[34]. Bsm DNA Polymerase can work actively at temperatures ranging from 60 to 68°C[34], but the enzyme works optimally at temperatures ranging from 59 to 62°C, according to the Thermo Fisher Scientific user guide[35]. This provides a framework for determining the amplification temperature during the optimization process. Amplification temperatures that are too low or too high can reduce enzyme activity, resulting in an insufficient amount of amplicon. This is indicated by the fainter of the DNA band on the electropherogram. A temperature of 61°C was also used to detect *Candida albicans* in suspected TB patient[36] and *Goose Circovirus* in goose liver[34]. From a laboratory perspective, the optimized Boil LAMP protocol offers advantages in terms of turnaround time, minimal equipment requirements, and reduced reagent costs compared to conventional molecular methods. These characteristics make the method suitable for use in primary health laboratories or peripheral settings with limited molecular diagnostic facilities.

In addition to the research results, there are limitations to this study. This study did not evaluate the sensitivity and specificity of the Boil LAMP method for detecting TB in saliva. Therefore, future studies should include more samples from patients with acute and latent TB, as well as people who have never been infected with *M. tuberculosis*. Furthermore, it is also recommended to compare the results of TB examination in saliva using the Boil LAMP method with the bacterial culture method, microscopic examination of sputum, and molecular rapid tests to determine the accuracy of the Boil LAMP method.

CONCLUSION

In conclusion, the modified boiling method using 200 µl chloroform, 300 µl isopropanol, and 1 ml of 70% ethanol effectively extracted salivary genomic DNA. Under these conditions, the LAMP assay optimally amplified the *gyrB* gene of *Mycobacterium tuberculosis* from saliva at a genomic DNA concentration of 100 ng/µl and an amplification temperature of 61°C. These findings demonstrate the feasibility of saliva-based Boil LAMP as a rapid and cost-effective molecular approach for TB detection, particularly in resource-limited laboratory settings.

AUTHORS' CONTRIBUTIONS

Patricia Gita Naully contributed to the conceptualization and methodology of the study, conducted the investigation and data curation, and prepared the original draft of the manuscript. Nur Andini Khoirunisa was involved in the methodology development, experimental investigation, data curation, and drafting of the manuscript. Sitti Romlah provided supervision and contributed to manuscript review and editing. All authors have read and approved the final version of the manuscript.

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DATA AVAILABILITY STATEMENT

The data supporting the findings of this study are available from the corresponding author upon reasonable request for academic and research purposes.

DISCLOSURE STATEMENT

The views and opinions expressed in this article are solely those of the authors and do not necessarily reflect the official policies or positions of their affiliated institutions. The data presented in this study are original, were generated by the authors, and have not been previously published or submitted for publication elsewhere.

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