THE EFFECT OF PRETREATMENT TEMPERATURE IN THE *Mycobacterium tuberculosis* EXTRACTION METHOD ON DNA QUALITY

Tri Yuli Setianingsih[*], Safira Fitri[**], Daniel Joko Wahyono[**], Vivi Setiawaty[*]

[*]*Department of Research, National Infectious Disease Center – Sulianti Saroso Hospital, Jakarta, Indonesia, triyuli.setianingsih@gmail.com, spu.rspiss@gmail.com*

[**]*Faculty of Biology, Universitas Jenderal Soedirman, Purwokerto, Indonesia, safira.fitri@mhs.unsoed.ac.id, danieljokowahyono@yahoo.com*

Email Correspondence : spu.rspiss@gmail.com

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Abstract: The existing DNA extraction process for *Mycobacterium tuberculosis* employs a pretreatment step that may not function at its full potential. This inefficient process led to complications in subsequent analysis; thus, it is necessary to determine the successful extraction method with or without adding a pretreatment step. This study aims to determine the effect of temperature pretreatment on the extraction method of *M. tuberculosis* on the quality of DNA for WGS examination. The research design was analytically descriptive with a kit-based extraction method from ExiPrepTM Dx Mycobacteria Genomic DNA Kit (K-4418), Bioneer. The results of this study were that DNA purity did not differ (p=0.959) between the pretreatment heating 95^0C (2.88) and no heating 95^0C (2.46). DNA concentration did not differ (p-value = 0.111) between pretreatment heating 95^0C (0.416 ng/µL) with no heating 95^0C (0.653 ng/µL). The relatively low DNA concentration resulted in the DNA bands not being visualized on agarose gel electrophoresis (0.8%). In conclusion, our study showed that the pretreatment heating step on *M. tuberculosis* DNA extraction does not affect DNA purity, concentration, and integrity. This inefficiency step can be discarded from the *M. tuberculosis* DNA extraction process to simplify the extraction processing. However, other extraction kit approaches need to be explored in future studies.
Keywords: DNA quality; *M. tuberculosis*; Temperature pretreatment; Sequencing

Abstrak: Proses ekstraksi DNA *Mycobacterium tuberculosis* yang saat ini dilakukan memerlukan *pretreatment* yang mungkin tidak berpengaruh secara optimal. Proses yang tidak efisiensi berpengaruh terhadap analisis selanjutnya, sehingga perlu untuk mengetahui keberhasilan metode ekstraksi dengan penambahan proses *pretreatment*. Tujuan penelitian ini adalah untuk mengetahui pengaruh pretreatment suhu pada metode ekstraksi *M. tuberkulosis* terhadap kualitas DNA untuk pemeriksaan WGS. Desain penelitian adalah deskriptif analitik dengan metode ekstraksi berbasis kit dari ExiPrepTM Dx Mycobacteria Genomic DNA Kit (K-4418), Bioneer. Hasil penelitian adalah kemurnian DNA tidak berbeda (p=0,959) antara pretreatment pemanasan 95^0C (2,88) dengan tanpa pemanasan 95^0C (2,46). Konsentrasi DNA tidak berbeda (p value = 0,111) antara pretreatment pemanasan 95^0C (0,416 ng/µL) dengan tanpa pemanasan 95^0C (0,653 ng/µL). Konsentrasi DNA yang relatif rendah mengakibatkan pita DNA tidak tervisualisasi pada elektroforesis gel agarosa (0,8%). Kesimpulan dari penelitian ini, bahwa langkah pemanasan awal pada ekstraksi DNA *M. tuberkulosis* tidak

berpengaruh terhadap kemurnian, konsentrasi, dan integritas DNA. Langkah ini dapat dihilangkan dari proses ekstraksi DNA *M. tuberkulosis* untuk menyederhanakan proses ekstraksi. Namun, pendekatan kit ekstraksi lainnya perlu dieksplorasi dalam penelitian selanjutnya.

Kata kunci: Kualitas DNA; *M.tuberkulosis*; temperatur *pretreatment*; sekuensing

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Introduction

Tuberculosis (TB) remains a serious global public health with over a billion deaths in the past 200 years. In 2022, a total of 1.3 million people died from TB *Mycobacterium tuberculosis* (*M. tuberculosis*), the etiological agent of TB, is estimated to have infected 1.7 billion people worldwide (WHO, 2023). TB diagnostic tests and technologies consist of automated and manual nucleic acid amplification tests which needed an extraction process of *M. tuberculosis* bacteria. While diagnostic test exists, their limitation necessitates the exploration of improved extraction processes for effective *M. tuberculosis* detection.

M. tuberculosis is a Gram positive obligate aerobic bacterium. The Mycobacterium genus has unique properties in the form of cell walls rich in lipids, a thick peptidoglycan layer containing mycolic acid, arabinogalactan, and lipoarabinomannan (Fauziah et al., 2020). *M. tuberculosis* is naturally resistant to antibiotics due to the presence of structures containing mycolic acid in the cell walls. The cell wall must be broken down to lyse the bacteria (Widodo et al., 2017).

DNA extraction is a series of processes separating DNA from other cell components such as proteins, carbohydrates, fats, etc. According to Sim et al., the main key to producing DNA samples that meet the criteria is at the DNA extraction stage. The accuracy of the extraction method used affects the quality of the DNA produced (Sim et al., 2015). According to Dayanti et al., DNA isolate is optimal if the absorbance ratio is 1.8-2.0 (Dayanti et al., 2019). Previously, related initial optimizations had been carried out at pretreatment temperature on DNA extraction of *M. tuberculosis* with manual methods. Pretreatment using heating at a temperature of 95^0C for 30 minutes results in optimal DNA quality.

DNA extraction method in *M. tuberculosis* needs to be added pretreatment heating at a temperature of 95^0C because this bacterium has a cell wall structure that contains mycolic acid, which is different from other bacteria. The results of this DNA extraction were used as a reference for further examination using the method of Whole Genome Sequencing (WGS) to encourage the use of genome data *M. tuberculosis* results from sequencing for precision medicine and improved effective care. This research aims to determine the effect of temperature pretreatment on the extraction method of *M. tuberculosis* on DNA quality for WGS examination.

Materials and Methods

Materials

This descriptive analytical study used a kit-based extraction method from ExiPrepTM Dx Mycobacteria Genomic DNA Kit (K-4418), Bioneer. The research was conducted at the Biomolecular Laboratory, RSPI Prof. Dr. Sulianti Soroso, Jakarta. This study used 30 bacterial liquid culture samples of Mycobacterium Growth Indicator Tube (MGIT) of *M. tuberculosis* obtained from Balai Besar Binomika (BB Binomika). The tools used include extraction tools, electrophoresis sets, Nanodrops, micropipettes, microtube, vortex, Bio Safety Cabinet (BSC), centrifuge, tubes Eppendorf, heat block, measuring cup, Erlenmeyer, Qubit 4 Fluorometer, Gel doc, microwave, and complete PPE. The materials used are bacterial culture samples (MGIT) *M. tuberculosis*, ethanol 70%, ExiPrepTM Dx Myobacteria Genomic DNA Kit, kit Qubit, agarose, loading dye, marker (1 kb plus from Invitrogen), ethidium bromide, and TAE 0.5x. This research consists of independent and dependent variables. The independent variable is pretreatment carried out with heating and without heating. The dependent variable is DNA quality, including purity, concentration, and integrity of bacterial *M. tuberculosis*.

Methods

Pretreatment Sample

A total of 3 mL of liquid culture sample (MGIT) was pipetted into a 1.5 mL tube (2 tubes). Centrifuge at 13,000 rpm for 10 minutes. The supernatant was discarded and washed with 1 mL of PBS 1x, homogenized. The sample was heated at 95^0C for 30 minutes using a heat block. The samples were cooled at room temperature for 5 minutes. Centrifuge at 13,000 rpm for 5 minutes. The supernatant was discarded, added with 400 µL of resuspension buffer, and continued with the ExiPrep extraction procedure TM Dx Mycobacteria Genomic DNA Kit (K-4418), Bioneer.

Bacterial DNA Extraction

Bacterial DNA extraction *Mycobacterium tuberculosis* following protocols from ExiPrepTM Dx Myobacteria Genomic DNA Kit (K-4418), Bioneer. The extraction process was carried out as follows: sample preparation, setup extraction process, sample pipetting, and extraction by operating the ExiPrep™16 Dx or the Existation™. Bacterial DNA is extracted automatically and put into an elution tube.

Test DNA Purity and Concentration

DNA purity was measured using the Nanodrop spectrophotometer (Thermo Scientific) at an absorbance ratio of 260/280 nm. Thawing DNA is to be measured at room temperature. The tools and materials were prepared, then the Nanodrop was turned on, and the nucleic acid part on the screen was pressed. The dsDNA option was selected, the plate to be used was selected, 2 µL of blank solution was put into plate 1, and then the samples were put sequentially into the plate. The nanodrop was inserted into the tool and then the button started pressed, waiting until the

results came out. After use, the nanodrop was cleaned using clean tissue and was stored in a dry place (Illumina DNA Prep Reference Guide, 2020).

Concentration measurement using the Qubit 4 Fluorometer method (Invitrogen) uses separate reagents which were homogenized first, then the sample was added. The working solution and the DNA sample were mixed until the total volume was 200 µL (sample volume 2 µL, working solution 198 µL). The mixture was vortexed for 2-3 seconds to prevent bubbles. The solution was incubated for 2 minutes at room temperature. Then it was read using the Qubit which was turned on to the program appropriate to the reagent used and waited until the results came out (Pratiwi et al., 2020).

DNA Integrity Test

The integrity of the extracted DNA was tested by electrophoresis using agarose gel with a concentration of 0.8% (Budiman et al., 2018). The steps for DNA electrophoresis are as follows: A total of 0.4 grams of agarose was weighed to be dissolved in 1x TAE buffer to a volume of 50 mL, then heated using a microwave until homogeneous. EtBr was added until homogenous to the agarose solution. After the gel had solidified, DNA samples with loading dye were loaded into the well. Electrophoresis was set to an 80 V voltage for 60 minutes. Once finished, the gel was removed and put into a gel doc and then the visualized DNA was observed (Fauziah et al., 2020)

Data Analysis

The research data obtained was analyzed using the software Statistical Product and Service Solutions (SPSS) using the Wilcoxon Test to compare the purity and concentration of DNA between sample pretreatment with heating 95^0C and without heating. The Wilcoxon test was carried out because the data was not normally distributed during the normality test. This statistical analysis was carried out using a significance level of 0.05 (α=5%).

Results and Discussion

This research was treated with pretreatment heating at 95^0C and without heating. This study's results show the DNA quality resulting from DNA extraction with the ExiPrep™ Dx Mycobacteria Genomic DNA (K-4418) kit, Bioneer with the principle magnetic beads. Table 1 shows the quality of DNA based on purity and concentration of DNA. Treatment results on pretreatment heating at 95^0C and without heating had an average DNA purity of 2.88 and 2.46, respectively. Optimal DNA purity is in the range of 1.8-2.0. DNA quality outside this value range indicates contamination, namely DNA purity <1.8 indicates protein, phenol, or other contamination that absorbs a wavelength of 280 nm. However, DNA purity >2.0 indicates RNA contamination (Wardana & Mushlih, 2021). This is thought to occur because Ribonuclease (RNAse) was not added during extraction. The extraction method in this study including the extraction kit and machine was fully

automation and closed system, so all extraction buffers were included from the kit manufacturer. Perhaps RNAse was not one of the components contained in the extraction kit. The research results of Sari et al. showed that using different extraction buffer solutions affected the DNA purity of each fungus isolate (Sari & Rosmeita, 2020). DNA is contaminated by RNA. This is because there is no addition of RNAse in the DNA extraction process. This RNAse is an enzyme that can degrade RNA to remove RNA from the DNA solution (Green & Sambrook, 2012)

DNA concentrations were measured with a Qubit 4 Fluorometer (Invitrogen). Treatment results on pretreatment heating 95^0C and without heating had an average concentration of 0.416 ng/µL and 0.653 ng/µL respectively. The DNA concentration resulting from this research was measured with a Qubit 4 Fluorometer (Invitrogen) because DNA sequencing with WGS requires accurate DNA concentration measurements. Qubit Fluorometer is a tool that uses the fluorescent dye principle, which is accurate, fast, and consistent to measure nucleic acid concentrations even though the concentration range is very small. Quantification using fluorescents is considered more sensitive than a spectrophotometer because it can measure the specific DNA of interest (Pratiwi et al., 2020).

The DNA concentration of the results of this research was at pretreatment heating 95^0C (0.055-2.190 ng/µL) and without heating (0.010-2.570 ng/µL). The DNA concentration obtained in this study was quite low compared to previous studies. When the DNA extraction process is not added lysis buffer will produce a low concentration of genomic DNA (Neves De Almeida et al., 2013). Cell dissociation was performed using heating 99^0C. In addition, using PBS during the extraction process allows little viral genomic DNA to be isolated so that the concentration obtained is low. The ideal DNA concentration is in the range of 5-100 ng/µl (Neves De Almeida et al., 2013).

Carried out DNA isolation of *M. tuberculosis* from MGIT culture heated at 95^0C for 15 minutes. A total of 20 MGIT culture samples obtained DNA concentration results measured by nanodrop, namely 0.5-840 ng/mL (Fauziah et al., 2020). Demonstrated DNA extraction of bamboo lobster gill tissue samples using the lysis buffer method. The sample was heated to a temperature of 950C for 10 minutes with the highest concentration value of 56.05 µg/mL (Adriany, D. T., 2020).

Shows the average DNA concentration obtained using the boiling method at a temperature of 95^0C for 10 minutes was 0.94 ng/µL, while DNA extraction using a kit obtained an average DNA concentration of 2.49 ng/µL (Falahul Ilmi et al., 2022). The difference in DNA concentration obtained from each sample can be determined by physical treatment and the ability of the extraction buffer to break down cells (Neves De Almeida et al., 2013). It is feared that heating could damage the target DNA if done for a long time (Muna et al., 2014). DNA extraction using different methods shows different concentrations and purity of DNA isolates

(Setiaputri et al., 2020). According to Lin et al, heat exposure can cause DNA to fragment. DNA strands can break randomly, making it difficult to extract DNA if physical or chemical treatment is given during the heating process (Lin et al., 2016).

Table 1. Results of Measurement of Purity and Concentration DNA of *M. tuberculosis*

Sample Code	DNA Purity (A260/A280 Ratio)		DNA Concentration (ng/µl)	
	Heating 95ºC	No Heating	Heating 95ºC	No Heating
1	1,67	2,00	0,327	0,362
2	1,97	1,47	0,377	0,111
3	1,92	2,06	0,335	0,069
4	1,91	2,07	0,077	0,051
5	2,27	2,29	0,088	0,010
6	3,06	2,44	0,093	0,010
7	12,00	3,00	0,212	0,232
8	3,18	8,36	0,083	0,232
9	2,38	2,64	0,055	0,229
10	2,06	1,83	0,332	2,060
11	2,30	2,56	0,588	0,399
12	1,53	1,97	0,372	0,192
13	2,00	1,90	0,375	0,431
14	1,98	2,56	1,430	0,531
15	2,09	1,90	0,453	2,410
16	2,10	2,04	0,308	2,570
17	2,02	2,15	2,190	2,060
18	1,67	2,00	0,275	0,742
19	1,97	2,32	0,686	1,030
20	9,25	2,39	0,320	0,287
21	2,06	2,70	0,449	0,493
22	2,24	2,25	0,935	1,330
23	2,36	2,28	0,155	0,347
24	2,03	2,25	0,717	1,730
25	2,60	2,31	0,423	0,227
26	5,00	3,29	0,194	0,458
27	2,50	2,40	0,199	0,397
28	2,56	1,96	0,186	0,287
29	4,45	2,54	0,086	0,174
30	1,40	1,82	0,152	0,135
Average	**2,88**	**2,46**	**0,416**	**0,653**

This study's electrophoresis results were that DNA bands were not visualized on agarose gel electrophoresis (0.8%) when samples were treated with pretreatment heating at 95°C and without heating (Figure 1). It was because the concentration of DNA was too low for almost well DNA loaded. Bands that are not visible can be

caused by the low concentration of extracted DNA. A good DNA quantity will show thicker, firmer, and more intact bands (Sundari, S. & Priadi, 2019). Qualitatively, the thickness of a DNA band represents its concentration (Fatchiyah et al., 2011). DNA band intensity from *M. tuberculosis* extracted using the heating method in bacteria, had the same results as the commercial kit-based DNA extraction method because no statistical differences were found. The DNA concentration of the sample produced during electrophoresis decreased after 15 minutes of heating and decreased over time after 30 minutes of heating (Adhyatma, I. G. R. et al., 2020).

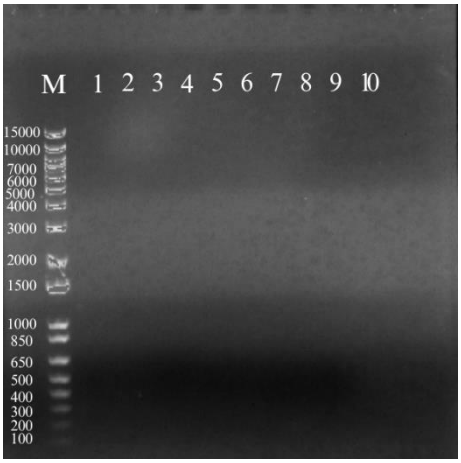

Figure 1. Visualization of Agarose Gel Electrophoresis Results (0.8%)
Description: (M) marker; (1-10) samples

Based on Emilia et al, electrophoresis of total DNA from wood samples cannot be interpreted because the DNA band results are not visible. The DNA concentration obtained ranged from 0.7 to 5.4 ng/µL with an average of 2.3 ng/µL. The Cetrimonium bromide (CTAB) method and the mercaptoethanol CTAB method did not show any clear DNA bands at all. The DNeasy Plant Mini Kit method only shows DNA bands in two samples and does not show DNA band results in other samples, so it cannot be interpreted. The DNA extraction yield is too low and cannot be visualized using UV techniques because the DNA is degraded into small fragments (Emilia & Anhar, 2021).

The results of this research were that DNA purity did not different (p value = 0.959) between pretreatment heating 950C (2,88) with no heating 950C (2.46). DNA concentration did not different (p value = 0.111) between pretreatment heating 95OC (0.416 ng/µL) without heating 95OC (0.653 ng/µL). The relatively low DNA concentration results in DNA bands not being visualized on agarose gel electrophoresis (0.8%). Therefore, pretreatment 950C does not affect the DNA purity and concentration of *M. tuberculosis*. The results of this research will be continued to the sequencing stage (WGS) because the WGS protocol with the Illumina kit provides a DNA purity tolerance of around 1.6-2.2 and a DNA

concentration of 0.03 ng/µL. This research needs to be continued with the development of DNA extraction methods using several other kits, either without pretreatment temperature or without kits with pretreatment temperature at a more efficient time to obtain optimal DNA concentration and purity for WGS examination.

Conclusion

Based on the results and discussion, it can be concluded that the pretreatment heating step on *M. tuberculosis* DNA extraction does not affect DNA purity, concentration, and integrity. This inefficiency step can be discarded from the *M. tuberculosis* DNA extraction process to simplify the extraction processing. However, other extraction kit approach needs to be explored in the future study.

Conflict of Interest

Tri Yuli Setianingsih is an employee of Sulianti Saroso Hospital and declares no conflict of interest. Safira Fitri is a student and Daniel Joko Wahono is a lecture in Jenderal Soedirman University. Vivi Setiawaty is a Director of Department Research, at Sulianti Saroso Hospital. All authors have no conflict of interest to declare.

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