

A SIMPLE METHOD OF DNA EXTRACTION OF *MYCOBACTERIUM TUBERCULOSIS* FROM SPUTUM CULTURES FOR SEQUENCING ANALYSISMaharani Pertiwi Koentjoro^{1*}; Adyan Donastin² and Endry Nugroho Prasetyo³

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*Corresponding Author's E-mail: maharani@unusa.ac.id**Article History**Received: 3rd Sept. 2020Revised Received: 23rd June 2021Accepted: 29th June 2021Published Online: 1st Sept 2021**Abstract**

Background: Concern has been raised about DNA extraction from *Mycobacterium tuberculosis* due to its complex procedure. This study demonstrates a simple and fast DNA extraction method of mycobacterial genome to subsequent molecular investigation, such as Polymerization Chain Reaction (PCR) amplification, with species-specific primers and sequencing.

Materials and Methods: Total DNA was isolated from *M. tuberculosis* cultured by using boil method. DNA was evaluated via measures of DNA quantity and quality (absorbance at 230, 260 and 280 nm), DNA integrity (electrophoresis). Molecular tests were tested namely PCR and sequencing.

Conclusions: The quality of DNA obtained is acceptable for PCR and sequencing analysis. These findings demonstrate that the method used is inexpensive and suitable for minimum infrastructure facilities.

Keywords: DNA extraction, *Mycobacterium tuberculosis*, sequencing, sputum

List of Abbreviations: ripA: Rpf-interacting protein A, TB: Tuberculosis, PCR: Polymerization Chain Reaction

Introduction

Tuberculosis (TB) is an infectious disease caused by *Mycobacterium tuberculosis* (Mingiano *et al.*, 2020). This bacterial infection is one of the top 10 causes of death worldwide. Furthermore, molecular analysis is a fast and sensitive diagnostic method for the detection and identification of these infections (Eddabra and Benhassou, 2018). High quality, pure DNA extraction protocol is a prerequisite for successful molecular investigations (Raschenberger *et al.*, 2016). The DNA extraction process for *M. tuberculosis* is often time-consuming, and requires specialized material or facilities (Almedida *et al.*, 2013). Therefore, faster and simpler methods for DNA isolation help to complete the process in a considerable amount of time, using only equipment needed to perform molecular investigation (Bjorn-Mortensen *et al.*, 2015). This study proposed a simple alternative protocol for performing DNA extraction of *M. tuberculosis* directly from Löwenstein-Jensen (LJ) medium, which consisted of cultures from patients diagnosed with full-blown TB. The effectiveness of the extraction method was assessed using Polymerization Chain Reaction (PCR) and sequencing assays.

Materials and Methods**Clinical data**

The sputum samples in this study were collected from patients undergoing treatment in the Hospital of Islam Jemursari (RSI), Surabaya-Indonesia, in the year 2020 (Table 1).

Table1: Clinical data from patients included the positive control.

No samples	Symptoms	Positive TB	Origin
1	Cough to clarify	Yes	Surabaya, Indonesia
2	Cough to clarify	Yes	Surabaya, Indonesia

The study was admitted by Institutional Ethics Committee. Informed patient consent was obtained and approved.

Growth of *Mycobacterium tuberculosis*

A total of 100 µL sputum samples were cultured in 5 mL of Löwenstein-Jensen culture medium and incubated at 37°C. H37Rv strain of *M. tuberculosis* were used as positive control (Cui *et al.*, 2012).

DNA extraction protocol and quantitative assessment

For each sample, a total of 0.5 mL UltraPure™ Distilled Water (Invitrogen) was added to 1.5 mL tube. A colony of *M. tuberculosis* cultures was transferred to the tube and mixed via a vortex. The suspension was placed in a boiling water bath (100°C) for 3 minutes to destroy the cell membrane. Then, suspension centrifuged at 15,000 rpm for 5 min in 4°C. A 300 µL supernatant was transferred to a sterile tube and processed for DNA analysis by spectrophotometry for evaluation of nucleic acid purity (ideal ratios: $A_{260}/A_{280} \geq 1.8$ and $A_{260}/A_{230} = 2$). The 1% agarose gel electrophoresis was used to evaluate DNA integrity.

Polymerization Chain Reaction (PCR) and electrophoresis

PCR was performed in a final volume of 50 µL containing 25 µL of GoTaq® Master Mixes (Promega); The ripA (Rpf-interacting protein A) gene in *M. tuberculosis* was amplified by ripA forward primers (5'-A TTCCGTCGGCTTTGAGTGT-3') and ripA reverse primers (5'-CGGCGGATCACGTA TTCAGA-3') (Koentjoro *et al.*, 2019). RipA gene is responsible for the cell division and cellular integrity of *M. tuberculosis* by cleave of the peptidoglycan layer connecting daughter cell (Maitra *et al.*, 2019). This gene expressed the endopeptidase ripA. In the absence of these genes, *M. tuberculosis* is unable to assemble the complex cell envelope. Therefore, ripA gene became the target of several anti-mycobacterial drugs. Amplification was carried out for 35 cycles, each consisting of initial denaturation at 92°C for 2 min, final denaturation at 92°C for 30 seconds, annealing at 92°C for 30 seconds, extension at 92°C for 1 minute, followed by a final extension at 92°C for 5 min. Additionally, PCR products were analysed by gel electrophoresis in 2% agarose gel. Target DNA fragments of 912 base pairs (bp) were viewed under UV light.

Sequencing analysis

For validation, ripA genes from PCR reaction were sequenced by Macrogen Inc (Korea) using Sanger sequencing method. Data sequences correlated with the specified sequences on the NCBI BLAST web service (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>).

Results

The DNA genome from *M. tuberculosis* were determined on 1 % agarose gel. Figure 1 shows the electrophoresis of DNA aliquots through heat extraction. Sharp bands and good resolution on agarose gel electrophoresis were observed, with no apparent signs of degradation. DNA quality and quantity are important parameters for a downstream molecular test.

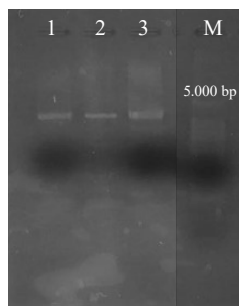


Figure 1: One percent (1%) Agarose gel electrophoresis of DNA aliquot. Lane 1 = *M. tuberculosis* H37Rv, Lane 2 = Sample 1, Lane 3 = Sample 2, M = Molecular marker 500 bp DNA

The integrity of nucleic acids was assessed on an agarose gel and the purity calculated by the ratio of absorbance at 260 and 280 nm (A_{260}/A_{280}) respectively, with values ≥ 1.8 indicating highly pure samples (Sellin *et al.*, 2014). Table 2 demonstrates A_{260}/A_{280} and A_{260}/A_{230} .

Table 2: Average sample absorbance

No samples	A_{260}/A_{280}	A_{260}/A_{230}	Concentration ($\mu\text{g}/\mu\text{L}$)
<i>M. tuberculosis</i> H37Rv	1.94	2.10	276
Sample 1	1.63	1.89	250
Sample 2	1.73	1.99	230

The aliquot was used as a DNA source for PCR, and the *ripA* gene was discovered to be successfully amplified (Figure 2, lanes 1-6). These findings indicate that the heat extraction method produces high-quality DNA from *M. tuberculosis* cultures samples for PCR.

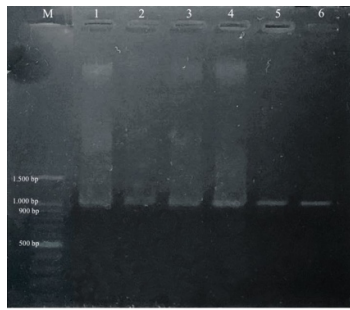


Figure 2: Two percent (2%) Agarose gel electrophoresis of *ripA* gene amplifications performed. Lane 1 = Molecular marker 100 bp DNA, Lane 1-2 = *M. tuberculosis* H37Rv, Lane 3-4= Sample 1, Lane 5-6 = Sample 2.

Moreover, the DNA samples from this method were used to perform nucleotide acid sequencing. Figure 3 shows that sequence alignment was generated through Sanger sequencing. Therefore, the results obtained show that sequence data have good matches with those in NCBI. This shows that DNA aliquot from heat-extraction is good for sequencing analysis.

Taxonomy	Number of hits	Number of Organisms	Description
☐ Mycobacterium	100	15	
• ☐ Mycobacterium tuberculosis complex	99	14	
• • ☐ Mycobacterium tuberculosis	72	14	Mycobacterium tuberculosis hits
• • • ☐ Mycobacterium tuberculosis variant bovis	9	9	Mycobacterium tuberculosis variant bovis hits
• • • • ☐ Mycobacterium tuberculosis variant bovis BCG	5	7	Mycobacterium tuberculosis variant bovis BCG hits
• • • • • Mycobacterium tuberculosis variant bovis BCG str. Tokyo 172	2	1	Mycobacterium tuberculosis variant bovis BCG str. Tokyo 172 hits
• • • • • Mycobacterium tuberculosis variant bovis BCG str. ATCC 35743	1	1	Mycobacterium tuberculosis variant bovis BCG str. ATCC 35743 hits
• • • • • Mycobacterium tuberculosis variant bovis BCG str. Korea 1168P	1	1	Mycobacterium tuberculosis variant bovis BCG str. Korea 1168P hits
• • • • • Mycobacterium tuberculosis variant bovis BCG str. Mexico	1	1	Mycobacterium tuberculosis variant bovis BCG str. Mexico hits
• • • • • Mycobacterium tuberculosis variant bovis BCG str. Moreau RDJ	1	1	Mycobacterium tuberculosis variant bovis BCG str. Moreau RDJ hits
• • • • • Mycobacterium tuberculosis variant bovis BCG str. Pasteur 1173P2	1	1	Mycobacterium tuberculosis variant bovis BCG str. Pasteur 1173P2 hits
• • • • • Mycobacterium tuberculosis variant bovis AF2122/97	1	1	Mycobacterium tuberculosis variant bovis AF2122/97 hits
• • • Mycobacterium tuberculosis variant caprae	1	1	Mycobacterium tuberculosis variant caprae hits
• • ☐ Mycobacterium tuberculosis variant africanum	2	2	Mycobacterium tuberculosis variant africanum hits
• • • • Mycobacterium tuberculosis variant africanum GM041182	1	1	Mycobacterium tuberculosis variant africanum GM041182 hits
• • • Mycobacterium tuberculosis variant microti	1	1	Mycobacterium tuberculosis variant microti hits
• Mycobacterium sp. 3/86Rv	1	1	Mycobacterium sp. 3/86Rv hits

Figure 3: BLAST analysis of *M. tuberculosis* H37Rv *ripA* gene PCR product sequences.

Discussion

To provide an efficient and low-cost method for the extraction of DNA from *M. tuberculosis* quantitatively and qualitatively for molecular investigation, the DNA extraction procedure was modified for *M. tuberculosis*. This is necessary due to the fact that not every laboratory has complete facilities and DNA extraction kits. Therefore, this new procedure was performed to obtain DNA from *M. tuberculosis* via boil method. The effect of heat treatment destroyed *M. tuberculosis* (Cardona *et al.*, 2015). Valenta *et al.* (2009) reported that heat lyses the cell membrane of *Mycobacterium smegmatis* which results in the release of DNA. Spectrophotometer was used to analyze DNA quantity and concentrations ranging from 276 µg/µL. The time required to complete the entire DNA extraction procedure was only 30 minutes. This result is relatively quick and simple than extraction method using kit (Aldous *et al.*, 2005).

Several studies had discovered a comparison method of extracting *M. tuberculosis* DNA. Miyata *et al.*, (2021) revealed that cetyltrimethylammonium bromide (CTAB) and thermolysis method was sufficient to extraction of *M. tuberculosis* DNA. These methods require 2 to 30 hours to obtained the DNA. Aldous *et al.*, (2005) reported that IDI lysis tubes showed the simple procedure, low cost, and time to require to complete the entire DNA extraction in less than 1 hours. Although very easy to do extraction using kit, it is expensive. Therefore, our method is promising to be applied in routine clinical practice.

This is a preliminary study with few samples. Furthermore, due to the costs of molecular tests in developing countries and the unavailability of diagnostic tools in every laboratory (Ssengooba *et al.*, 2015), this simple method used to extract DNA of *M. tuberculosis*, becomes a viable alternative. This extraction procedure would be ideal for laboratories in Biosafety Level-3 (BSL-3) (Ssengooba *et al.*, 2015), and laboratory staff will be safe at work with lesser exposure time with *M. tuberculosis*. Additionally, this result proposes a suitable method for other forms of molecular analysis.

Conclusion

It is concluded that the DNA extraction protocol employed for *M. tuberculosis* from clinical sputum culture in the present study was useful and successful for performing molecular analysis such as PCR amplification and sequencing with species-specific primers.

Additionally, the protocol is useful in laboratory settings with minimal infrastructure, less reagents and reduction in the risk of infection to the laboratory staff.

Conflict of interests

The authors declare that there is no conflict of interest associated with this study.

References

1. Aldous, W.K., Pounder, J.I., Cloud, J.L. and Woods, G.L. (2005). Comparison of six methods of extracting *Mycobacterium tuberculosis* DNA from processed sputum for testing by quantitative Real-Time PCR. *Journal of Clinical Microbiology*; **43(5)**, 2471–2473, doi:10.1128/JCM.43.5.2471–2473.2005.
2. Bjorn-Mortensen, K., Xallet, J., Lilebaek, T., Andersen, A. B., Niemann, S., Rasmussen, E.M. and Kohl, T.A. (2015). Direct DNA extraction from *Mycobacterium tuberculosis* frozen stocks as a reculture-independent approach to whole-genome sequencing. *Journal of Clinical Microbiology*; **53(8)**, doi: 10.1128/JCM.00662-15.
3. Cardona, P., Marzo-Escartin, E., Tapia, G., Díaz, J., García, V., Varela, I., Vilaplana, C. and Cardona, P. J. (2016). Oral Administration of Heat-Killed *Mycobacterium manresensis* Delays Progression toward Active Tuberculosis in C3HeB/FeJ Mice. *Frontiers in microbiology*; **6**, doi.org/10.3389/fmicb.2015.01482.
4. Cui, Z., Wang, J., Zhu, C., Huang, X., Lu, J., Wang, Q., Chen, Z., Wang, J., Zhang, Y., Gu, D., Jing, L., Chen, J., Zheng, R., Qin, L., Yang, H., Jin, R., Liu, Z., Bi, A., Liu, J. and Hu, Z. (2012). Evaluation of a novel biphasic culture medium for recovery of mycobacteria: a multi-center study. *PloS one*; **7(4)**, e36331, doi.org/10.1371/journal.pone.0036331.
5. de Almeida I. N., Carvalho W. D. S., Rossetti M. L., Costa, E. R. D. and de Miranda S. S. (2013). Evaluation of six different DNA extraction methods for detection of *Mycobacterium tuberculosis* by means of PCR-IS6110: preliminary study. *BMC Research Notes*; **6(1)**, doi: 10.1186/1756-0500-6-561.
6. Eddabra R. and Benhassou H.A. (2018) Rapid molecular assays for detection of tuberculosis. *Pneumonia*; **10(1)**, doi: 10.1186/s41479-018-0049-2.
7. Koentjoro, M. P. and Prasetyo, E. N. (2019). Bioinformatics as a preliminary method for endopeptidase peptidoglycan procurement analysis of *Mycobacterium tuberculosis*. *Seminar Nasional Teknologi dan Sains*; pp.41–49.
8. Maitra, A., Munshi, T., Healy, J., Martin, L. T., Vollmer, W., Keep, N. H. Keep, and Bhakta, S., (2019). Cell wall peptidoglycan in *Mycobacterium tuberculosis*: An Achilles' heel for the TB-causing pathogen. *FEMS Microbiology Reviews*; **43(5)**, 548–575, doi: 10.1093/femsre/fuz016.
9. Miggiano, R., Rizzi, M. and Ferraris, D. M. (2020) *Mycobacterium tuberculosis* pathogenesis, infection prevention and treatment. *Pathogens*; **9(5)**, doi: 10.3390/pathogens9050385.
10. Miyata, M., Santos, A.C.B., Mendes, N.H., Cunha, E.A., de Melo, F.A.F. and Leite, C.Q., (2011). Assessment of the quality of DNA extracted by two techniques from *Mycobacterium tuberculosis* for fast molecular identification and genotyping. *Genetics and Molecular Microbiology*; **42(2)**, <https://doi.org/10.1590/S1517-83822011000200045>.
11. Raschenberger, J., Lamina, C., Haun, M., Kollerits, B., Coassin, S., Boes, E., Kadenko, L., Kootagen, A. and Kronenberg, F. (2016). Influence of DNA extraction methods on relative telomere length measurements and its impact on epidemiological studies. *Scientific Report*; **6(1)**, doi: 10.1038/srep25398.
12. Sellin, J. M. K., Kiss, A.J., Smith, A.W. and Oris, J.T. (2014) A comparison of commercially-available automated and manual extraction kits for the isolation of total RNA from small tissue samples. *BMC Biotechnology*; **14(94)**, doi:10.1186/s12896-014-0094-8.
13. Ssengooba, W., Gelderbloem, S.J., Mboowa, G., Wajja, A., Namaganda, C., Musoke, P., Mayanja-Kizza, H. and Joloba, M.L. (2015). Feasibility of establishing a biosafety level 3 tuberculosis culture laboratory of acceptable quality standards in a resource-limited setting: an experience from Uganda. *Health Research Policy and System*; **13(4)**, doi:10.1186/1478-4505-13-4.
14. Valente, W., Pienaar, E., Fast, A., Fluitt, A., Whitney, S., Fenton, R., Barletta, R., Chacon, O. and Viljoen, H. (2009). A kinetic study of in vitro lysis of *Mycobacterium smegmatis*. *Chemical Engineering Science*; **64(9)**, 1944–1952, doi.org/10.1016/j.ces.2008.12.015.