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Research Article

Evaluation of Methods for Extracting DNA of *Mycobacterium Tuberculosis* from Sputum Specimens and Bronchial Washing Fluids

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Abstract

Tuberculosis is one of the most infectious diseases causing high death rates and serious public health impact. Common TB diagnostic methods such as chest X-ray, sputum smear and culture have limited sensitivity, specificity, and time-consuming compared with molecular biology techniques. In this study, five methods of DNA extraction of *Mycobacterium tuberculosis* (MTB) from 25 AFB-positive sputum samples and 25 bronchial washing fluid samples were compared and evaluated the extraction efficiency. The DNA products were then performed to a polymerase chain reaction (PCR) to amplify the IS6110 and 16S rRNA genes. The results have successfully optimized the DNA extraction method of MTB by 2ndmethod, it's using Lysis buffer (including: Tris-HCl 1M, pH 8.5; EDTA 0.5M; SDS 10%; and NaCl 5M). The obtained DNA product has high purity and integrity and the PCR reaction was successfully performed. This result creates a premise for building a real-time 16S rRNA test procedure in the diagnosis of pulmonary tuberculosis in a few medical facilities in Hanoi.

Keywords: Tuberculosis, Sputum, DNA Extraction, *Mycobacterium tuberculosis*

Introduction

Tuberculosis, a disease caused by the *Mycobacterium tuberculosis* bacteria, has been known for centuries and remains a highly fatal infectious disease, ranking 13th on the list of disease causes of death[1]. In 2020, 1.5 million lives were lost to the disease, including 214,000 people with HIV-induced immunodeficiency. Vietnam is among the 30 countries with the highest burden of TB in the world, with an estimated 124,000 new cases each year. Tuberculosis is an infectious disease caused by the tuberculosis bacteria *M. tuberculosis* complex, which includes the species *M. tuberculosis*, *M. canetti*, *M. microti*, *M. africanum*, *M. canetti* and *M. bovis*. Mycobacteria are non-motile, non-spore-forming, aerobic rod-shaped bacteria that are 2-4 µm in length and possess a lipid-rich cell wall that confers "antacid" properties and make them resistant to many substances sterilization and antibiotics [2].

The current vaccine for TB only offers limited protection against pulmonary TB infections. Besides, the long-term treatment regimens with anti-tuberculosis drugs can cause side effects of medications and contribute to the development of antimicrobial resistance. Conventional methods of diagnosis, such as clinical manifestations, sputum tests, culture samples, or chest X-rays, can prolong the diagnosis period and impact disease detection and treatment[3].To address these challenges, molecular diagnostics in TB offer a fast and accurate method for diagnosing *M. tuberculosis*, especially for samples with low bacterial concentrations. However, the efficiency and sensitivity of the method depend on the status of samples, particularly in early diagnosis when the number of bacteria is low.

In the study, to improve the efficiency of molecular diagnostics, we assessed and compared five methods for extracting DNA from TB sputum and bronchial washing fluids. Despite the challenges due to the thick and complex cell wall of *M. tuberculosis*, our study aims to supplement the effectiveness of molecular diagnostic methods for TB.

Materials and Methods

Ingredient

A total of 25 sputum samples and 25 bronchial washing fluids samples were obtained from patients suspected of being infected with Mycobacterium tuberculosis at the Center for Internal Respiratory Medicine, 103 Military Hospital. The samples were designated with numbers ranging from 1 to 25 and were confirmed to be positive for Acid Fast Bacillus (AFB). All samples were collected in accordance with the medical ethics regulations that were approved in the research project code NVCC40.02/23-24 by the Senior Research Support Program of Vietnam Academy of Science and Technology.

Chemical reagents for PCR (Thermofisher Scientific, USA), CTAB (PanReac AppliChem, Italy), NaOH (Sigma Aldrich, USA), N-acetyl - cysteine (Sigma Aldrich, USA), Tris-HCl (Bio Basic, Canada)), EDTA (Thermofisher Scientific, USA), SDS (Sigma, USA), PBS (Thermofisher Scientific, USA), Ethanol (Invitrogen, USA), Lyzozyme (Thermofisher Scientific, USA), Proteinase K (Thermofisher Scientific, USA), Phenol - Chlorofrom-Isoamyl alcohol (Thermofisher Scientific, USA), Isopropanol (Invitrogen, USA), CH3COONa, PBS (Phosphate buffered saline) (Invitrogen, USA), QIAamp DNA Mini Kit (QIAGEN, Germany). The primer pair amplifying the *IS6110* gene is specific for TB bacteria and *16S rRNA* gene primers (Integrated DNA Technologies, IDT, USA).

Method of Handling Patient Samples

The homogenize sputum samples: The samples were initially treated with PBS buffer and subsequently centrifuged to eliminate the viscous solution and gather the residue. Add PBS buffer, use the Sonicate digester to homogenize the specimen. Add buffer solution TE (Biotech Grade), SDS 10%, Protease K; then incubated at 56^{o}C for one hour with shaking at 600 round per minute.

Using Cetrimonium Bromide (CTAB) and N-acetyl - cysteine - NaOH: Sputum specimens were centrifuged at high speed to remove the supernatant. Then continue to be treated with 0.05M NaOH buffer + 0.02% CTAB with the addition of NALC. The suspension was then heated at 96 – 100oC for 30 minutes to kill *M. tuberculosis*. Continue to centrifuge the treated sputum at 1500 round per minute for 15 minutes, collect the residue.

For bronchial washing fluid samples, centrifuge at 10000 round per minute for 10 minutes, collect the residue.

Total DNA Extraction Method

*Method 1:*Tris – EDTA supplemented with 10% SDS and ProteinaseK[4]:

Samples were treated according to a previously established protocol. Tris-EDTA buffer and 10% SDS were added to the sample, followed by Proteinase K. The mixture was heated at 56^{o}C for 1 hour with shaking at 600 rounds per minute. Phenol-chloroform-isoamyl alcohol (25:24:1) was added at a 1:1 volume ratio to the liquid in the tube. The solution was gently inverted to ensure homgenity and then centrifuged to collect the DNA in the suspension. The DNA fraction was precipitated with Isopropanol and CH3COONa 5M followed by incubation at -20^{o}C. The resulting DNA fraction was then washed with 70% Ethanol and dissolved in distilled water (UltraPure Distilled Water, Invitrogen, USA) before quality assessment by 1% Agarose gel electrophoresis and NanoDrop spectrophotometers.

Method 2: Lysis buffer

Lysis buffer was prepared according to the formula: 1 mL Tris-HCl 1M, pH 8.5; 0.1 mL EDTA 0.5M; 0.2 mL SDS 10%; 0.4 mL 5M NaCl in 10 mL autoclaved distilled water[5]. The treated samples were added with Lysis buffer and Lysozyme (10 mg/mL) followed by incubation on ice for 30 minutes to resolve the cell membrane of

M. tuberculosis.20 µL ProteinaseK was added, and the mixture was incubated at 56°C for 3 hours. Phenol-chlorofrom-isoamylancohol (PCI, 25:24:1) was added and the mixture was centrifuged at high speed to collect the DNA fractions. The resulting DNA fraction was then washed with 70% Ethanol and dissolved in distilled water (UltraPure Distilled Water, Invitrogen, USA) before quality assessment by 1% Agarose gel electrophoresis and NanoDrop spectrophotometers.

Method 3: Cetrimonium Bromide (CTAB) – NaCl

The sample was dissolved in Tris-EDTA buffer, pH 7.6 and brought to a final volume of 1 mL. Lysozyme (10 mg/mL) was added and incubated at 37°C for one hour. The suspension was then supplemented with CTAB-NaCl (50 µl of 5M NaCl and 40 µl of 10% CTAB)[4] and incubated for 10 minutes at 65°C. Chloroform-isoamyl alcohol (24:1) was added to form a homogeneous mixture. The DNA was precipitated with Isopropanol and CH3COONa 0.3M, followed by incubation at -20°C for at least 3 hours. Centrifuge for 15 min at 14,000 round per minutefor 20 min at 4°C to collect the precipitated DNA. Wash the precipitate again with 70% cold ethanol. The resulting DNA fraction was then washed with 70% Ethanol and dissolved in distilled water (UltraPure Distilled Water, Invitrogen, USA) before quality assessment by 1% Agarose gel electrophoresis and NanoDrop spectrophotometers.

*Method 4:*N-acetyl-cysteine[6]:

The sample is treated with N-acetyl - cysteine - NaOH. Prepare Extraction buffer (50 mMTris-HCl, 25 mM EDTA, 5% mono-sodium glutamate, pH 7.4). Samples after processing are diluted with Extraction buffer. Add Lysozyme 10 mg/mL, incubate at 37°C for 2 hours, with gentle stirring 1-2 times during incubation. Proteinase K was added and incubated overnight (16 hours) at 45°C. Continue adding Phenol-chlorofrom-isoamylancohol (25:24:1), then centrifuge at high speed to collect the DNA fractions. The DNA was precipitated with Isopropanol and CH3COONa 0.3M, followed by incubation at -20°C for at least 3 hours. Centrifuge for 15 min at 14,000 round per minute for 20 min at 4°C to collect the precipitated DNA. Wash the precipitate again with 70% cold ethanol. The resulting DNA fraction was then washed with 70% Ethanol and dissolved in distilled water (UltraPure Distilled Water, Invitrogen, USA) before quality assessment by 1% Agarose gel electrophoresis and NanoDrop spectrophotometers.

*Method 5:*QIAmp Blood and Tissue kit[7]

Samples were treated with a lysis buffer consisting of Lysozyme (10 mg/mL) and incubated for 30 minutes at 37°C. Proteinase K and buffer AL (without ethanol) were added and incubated for 3 hours at 60°C. Ethanol was added, and the sample was centrifuged. The DNA was purified using a DNAeasy Mini spin-column tube and eluted with buffer AE. The resulting DNA fraction was washed with AW1 and AW2 buffers before being dissolved in distilled water for quality assessment by 1% agarose gel electrophoresis and NanoDrop meter.

Measure DNA Product Concentration

The concentration of DNA products was quantified through utilization of a NanoDrop™ Lite Spectrophotometer (Thermofisher Scientific, USA). The absorption readings were measured at 260 nm and recorded directly on the spectrophotometer display. Replicates were conducted for each sample in triplicates. The acquired data were subsequently transferred to a SPSS software to compute the standard deviation. The resultant standard deviation value for DNA concentration was determined to be ± 0.076 ng/µL, while the standard deviation for OD value is± 0.03.

PCR Amplification Method

The PCR reaction was performed using the primer pairs shown in Table 1. The primer pairs were used to amplify the bacterial *16SrRNA* gene region[8] and the TB *IS6110* gene region[9].

Table 1. Amplification target, primer and primer temperature for PCR

Target	Primer pair name and sequence	Primer attachment temperature	Size
16S rRNA	341F: 5CCTACGGGAGGCAGCAG3	55°C	567
	907R: 5CCGTCAATTCCTTT3		
IS6110	IS6110 F: 5CCTGCGAGCGTAGGCGTCGG3	64°C	123
	IS6110 R: CTCGTCCAGCGCCGCTTCGG3		

PCR reaction was performed with a volume of 25µL including 10.25µL of 2X distilled water; 2.5µL 10X PCR buffer; 2.5µL $MgCl_2$ 25mM; 2.5µLdNTP 2.5mM; 1µL each primer (341F, 907R or IS6110F, IS6110R) 50 pmol/µL; 0.25µLTaq DNA Polymerase 5 U/µ; 5µL of total DNA sample and according to the following thermal cycles: 01 cycle of 95°C/ 5 minutes; 35 cycles (95°C/ 30 sec; 50-57°C / 1 min; 72°C / 1 min); 01 cycle of 72°C/ 2 minutes; kept at 4°C until analysis, for the *IS6110* fragment amplification reaction and 01 cycle of 95°C /5 min; 35 cycles (95°C / 30 sec; 57°C / 1 min; 72°C / 1 min); 72°C /2 minutes; kept at 4°C until analysis, for *16S rRNA*gene amplification.

Results and Discussion

Early diagnosis of *M. tuberculosis* plays an important role in controlling the progression of the disease. Applying PCR amplification to detect genetic traces of tuberculosis bacteria directly from patient samples has been shown to be effective [10]. The sensitivity of PCR reaction depends on the quality of the extracted DNA, especially when lysis of the cell wall of *M. tuberculosis* is difficult due to the large amount of polysaccharide layer[11]. Therefore, the use of conventional membrane lysis methods applied to Gram-positive bacteria is not appropriate, due to the quality of extracted DNA products having low concentration and poor purity. In this study, we conducted and evaluated the effectiveness of five different sample treatment and extraction methods.

DNA extraction using Lysis Buffer (2nd method) and gene amplification

DNA isolated from *M. tuberculosis* strains from sputum samples was subjected to agarose gel electrophoresis (as depicted in Figure 1) for the purpose of assessing its quality and quantity. Purified DNA was utilized as a template for PCR amplification of two genes: the *16S rRNA* gene using primer pairs 341F and 907R, and the *IS6110* gene using primer pairs IS6110F and IS6110R.

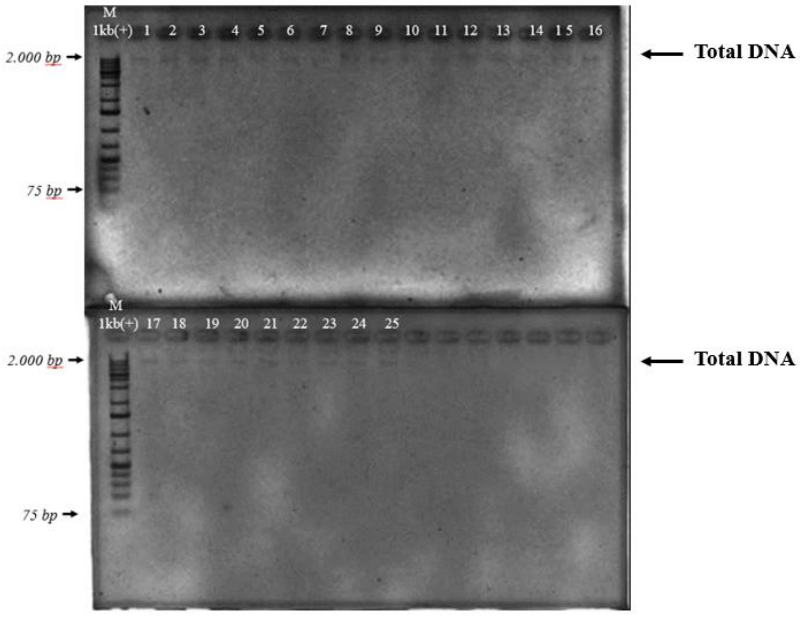

Figure 1. Electrophoresis was utilized to evaluate the entire DNA yield obtained from sputum samples. M: DNA standard scale 1kb+; 1-25: The corresponding extracted samples were received from Military Hospital 103.

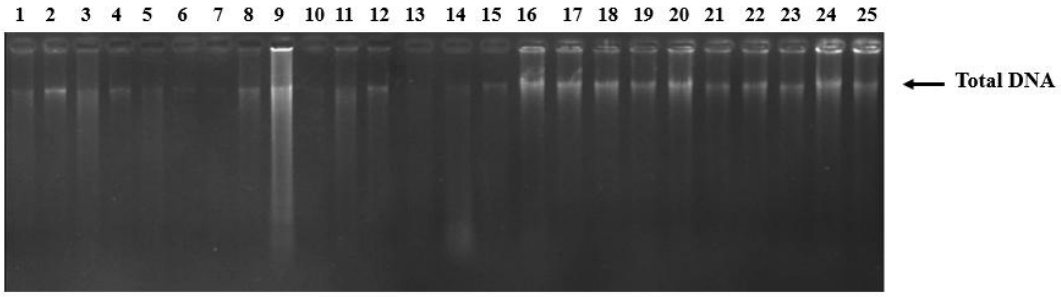

Figure 2. Electrophoresis was utilized to evaluate the entire DNA yield obtained from bronchial wash samples. No 1-25: The corresponding extracted samples were received from Military Hospital 103.

The electrophoretic analysis of the PCR product on 1% agarose gel (Figure 3, 4) yielded a distinct DNA band of precise size measuring 567 base pairs, which corresponds to the targeted size of the short *16S rRNA* fragment in bacteria. This outcome unequivocally confirms the success of the experimental design.

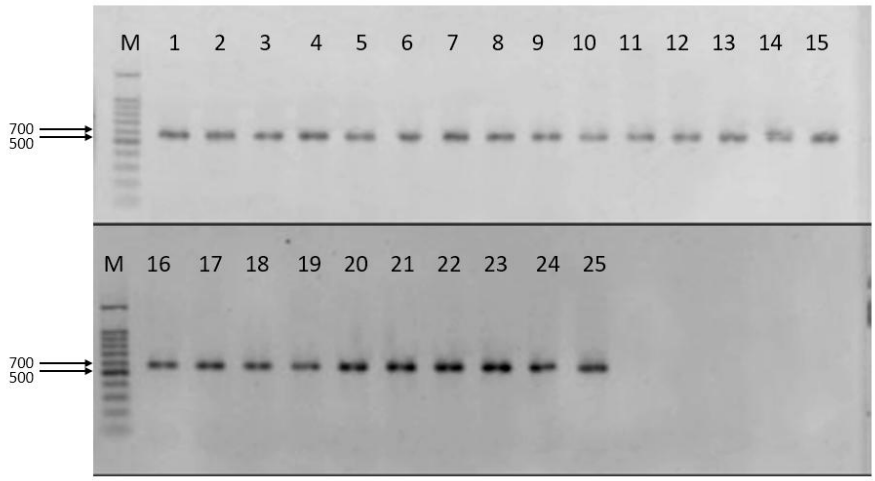

Figure 3. Electrophoresis of *16S rRNA* gene fragment (from sputum samples). PCR products using primers 341F and 907R. M: DNA standard scale 1kb+; No 1-25: The corresponding extracted samples were received from Military Hospital 103.

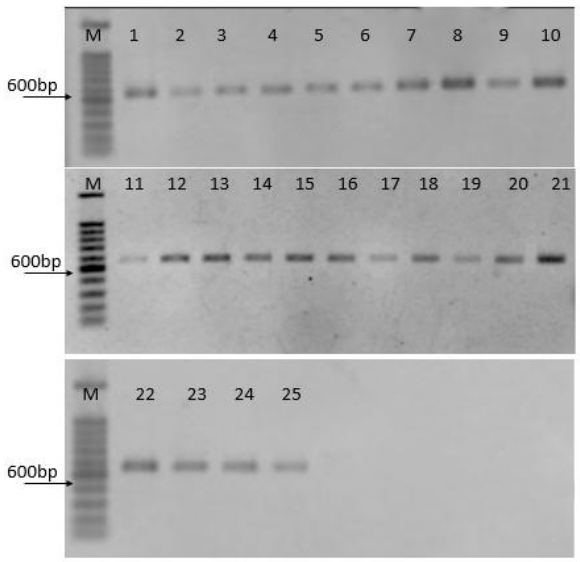

Figure 4. Electrophoresis of *16S rRNA* gene fragment (from bronchial wash samples). PCR products using primers 341F and 907R. M: DNA standard scale 1kb+; No 1-25: The corresponding extracted samples were received from Military Hospital 103.

To determine exactly that the extracted DNA is from TB bacteria, PCR is performed with the primer pairs IS6110F and IS6110R to amplify the specific IS6110 insertion sequence in TB bacteria. Electrophoresis results showed a band with a size corresponding to the *IS6110* gene (~123bp) (Figure 5, 6). For DNA samples isolated from bronchial fluid, PCR reaction targeted at the *IS6110* gene was successfully performed on 12 out of 25 samples. It is concluded that the remaining 13 samples may not contain TB bacteria. However, it is certain that the *IS6110* gene fragment has been amplified and succeeded in extracting DNA from TB bacteria in the patient samples.

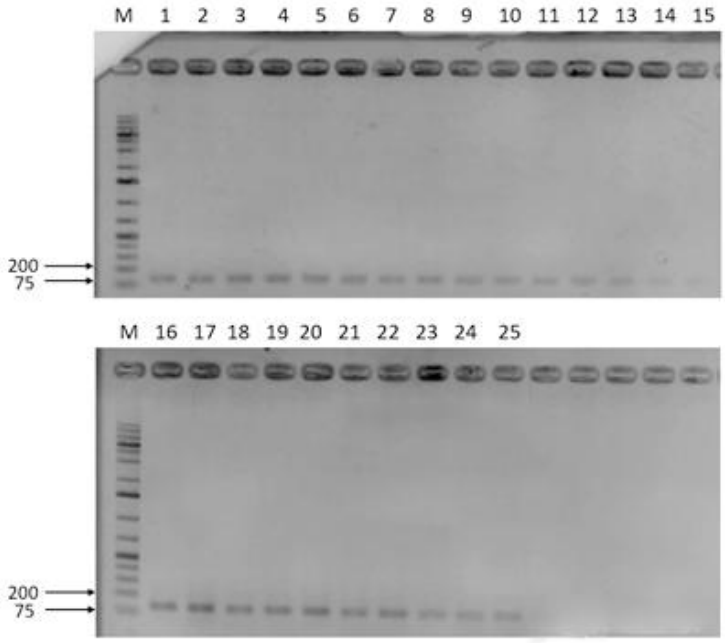

Figure 5. Electrophoresis of the PCR product (from sputum samples) of the *IS6110* gene fragment using the primer pairs IS6110F and IS6110R. M: DNA standard scale 1kb+; 1-25: The corresponding extracted samples were received from Military Hospital 103.

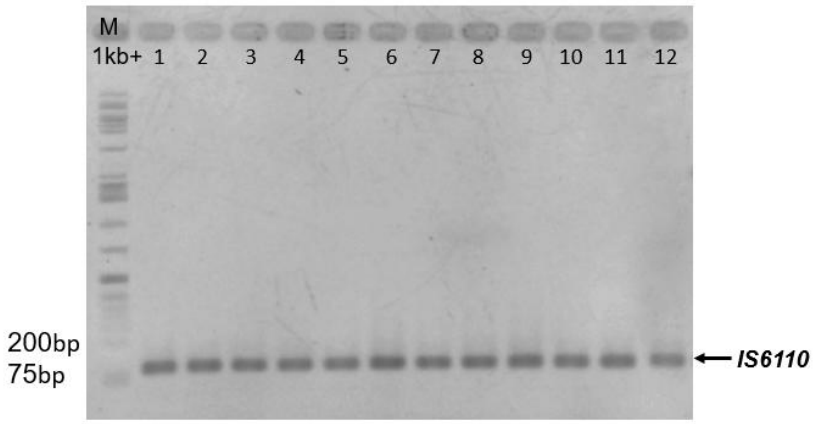

Figure 6. Electrophoresis of the PCR product (from bronchial washing fluid samples) of the *IS6110* gene fragment using the primer pairs IS6110F and IS6110R. M: DNA standard scale 1kb+; 1-12: The corresponding extracted samples were received from Military Hospital 103.

DNA Extraction by Other Methods

DNA isolated from strains of M. tuberculosis on patient samples by other methods resulted in ineffective extraction. The results of electrophoresis to check total DNA (Figure 7) showed that the remaining sample processing methods could not resolve all the polysaccharides as well as the residues from the patient samples, affecting the efficiency of DNA extraction.

Figure 7. Electrophoresis of the extracted total DNA product by other sample processing methods. M: DNA standard scale 1kb+; 1-5: the corresponding extracted samples were received from Military Hospital 103.

Carrying out PCR reaction to check extracted DNA samples with *16S rRNA* primers with primer pairs 341F and 907R, the results of electrophoresis test (Figure 8) showed no bands. Therefore, it is not possible to extract enough DNA to perform PCR reaction.

Figure 8. Electrophoresis of *16S rRNA* gene fragment PCR products using primers 341F and 907R. M: DNA standard scale 1kb+; 1-5: the corresponding extracted samples were received from Military Hospital 103.

Regarding ultrasonic sample treatment, all extraction methods results indicated that the DNA total samples were still viscous and very low concentration. Additionaly, the PCR reaction performed no band or many bands appear incorrect design size. Once of reason that the outer membrane structure of *M. tuberculosis* is composed of peptidoglycan, arabinogalactan and mycolic acid, these make it difficult to disrupt the membrane cell structure to release DNA total [11]. Besides, the glycolipid membrane, lipoglycan and non-polar lipids that combine with the cell wall layer to form a durable structure, when ultrasound waves break down this cell wall layer, the polysaccharides and lipids are not lysed, causing low efficiency in DNA extraction[11].

The use of N-acetyl - cysteine to dissolve mucus in sputum specimens[6], and using of CTAB to remove polysaccharides and lipids [12]have been successfully applied in previous studies. However, when applying the above methods in here, the polysaccharide parts were not completely resolved, resulting in the total DNA always leaving a long viscous streak on the run of 1% agarose electrophoresis gel. The amount of DNA obtained during the extraction stage using PCI very low, making PCR reactions impossible.

By applying a combination of sample treatment with CTAB and N-acetyl – cysteine – NaOH combined with cell

membrane lysis methods using SDS[13], all proteins were treated with Proteinase K and lysozyme enzymes also added to support the process of cell membrane lysis, so the resulting DNA product meets the requirements for purity and concentration and can be used to perform PCR reactions.

Conclusion

Taken together, total TB DNA extraction and amplification of the TB specific gene sequence have been successfully performed. Using Lysis Buffer (2nd method) to DNA extraction and through PCR reaction with target *IS6110* gene of *M. tuberculosis* can be effective in identifying MTB from sputum samples and bronchial wash samples.

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Ethics Approval

All content experiments received the approval ethical issues of Vietnam Academy of Science and Technology; Institute of Genome Research; Approval No.: 2432/QD-VHL; Date: 2022-12-28). All informed consent was obtained from the study participants. And they were also conducted and designed in line with the Declaration of Helsinki or other comparable ethical standards (Vietnam Military Medical Academy; Military Hospital 103; Approval No.: 04/2022/CNChTY-HDDD; Date: 2022-12-12).

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