Comparison of The Use of KOH and NaOH in Deproteination of Sputum Samples for the Isolation of *Mycobacterium tuberculosis* DNA

Fredrik Abia Kande[a*], B Saputra[b], [a]Faculty of Teacher Training and Education, Universitas Tribuana Kalabahi, Indonesia, [b]Faculty of Teacher Training and Education, Universitas Lampung, Indonesia.

Tuberculosis is a disease that infects the lungs, caused by the bacterium Mycobacterium tuberculosis. In general, the diagnosis of microscopic examination can be made in this disease, but there are still many weaknesses. This research encourages comparing two compounds that have the ability to dissolve, neutralize, and deprotote sputum samples for the isolation of DNA Mycobacterium tuberculosis, namely NaOH and KOH. Sputum samples used were 15 positive samples examined by the Community Health Center and hospital. Tuberculosis diagnosis in a microscopic smear examination, then DNA, PCR, and electrophoresis were isolated by pre-treatment deproteination before using 1N NaOH solution and 0.5N KOH solution. DNA bands can be seen on the electrophoresis results measured using Image J. The results of This research shows that the isolation of DNA from sputum samples using KOH is better than the results of isolation of sputum samples using NaOH.

Key words: *DNA isolation, smear microscopy, Mycobacterium tuberculosis*

Introduction

In 2007, pulmonary tuberculosis (pulmonary tuberculosis) was estimated to have reached 13.7 million active chronic cases at the global level while in 2010, an estimated 8.8 million new cases were added, and 1.5 million deaths, the majority of which occurred in developing country. According to WHO in 2010, Indonesia was ranked fifth with the number of TB patients at 429,000 people or 5.8% percent of the total number of tuberculosis in the world. Generally caused by lack of awareness of sanitation and hygiene (World Health Organization, 2013).

Mycobacterium tuberculosis is a bacterium that causes tuberculosis (Kumar *et al*., 2010; Taufiq, 2007; Kent and Kubica, 1985). The bacteria are rod-shaped which are resistant to acids (Valde's, Pose, San, and Martinez, 2003; Berger and Meiia, 1973). In general, laboratory tests for diseases caused by bacteria are stained Ziehl-Neelsen smear method which is read microscopically (Ferrer, 1997). The gold standard for the examination of Mycobacterium tuberculosis is culture on Lowenstein Jensen's medium. However, it is

suspected that there are still weaknesses, including the time required to diagnose the disease. BTA microscopic examination is also carried out to diagnose TB disease, but often errors in reading and the quality of preparations have an effect on the results of the diagnosis so that it will cause false positive and negative results (Jawetz, Melnick, Adelberg, 2007; Seibert, Haynes, Middleton, and Bass, 1999).

At this time, the application of biotechnology in the health sector is starting to develop rapidly. DNA isolation techniques, PCR (Polymerase Chain Reaction) and electrophoresis are widely used in several countries to diagnose various diseases (Fatchiyah, 2011; Moon, Chang, and Kim, 2005; Nagesh, Sehgal, Jindal, and Arora, 2001). This is because biotechnology applications have a higher level of sensitivity in detecting a disease. For example, to examine the isolation of DNA Mycobacterium tuberculosis, sputum (phlegm) samples are used in small volumes and with a faster time but provide accurate results (Mishra, Singhal, Chauhan, Katoch, and Srivastava, 2005; Gomez et al., 2012). It is this progress and development that will help in determining the steps to be taken in overcoming various health problems (Fatchiyah, 2011; Mirza, Restrepo, McCormick, and Fisher-hoch, 2003).

In the process of DNA isolation, sodium hydroxide (NaOH) is used for the neutralization step of acidic sputum samples, sputum samples are made in a neutral state so as not to degrade DNA. Then NaOH is also used for the pre-deproteination stage, namely removing the protein or dissolving the protein in the sputum sample before entering the DNA isolation stage. NaOH can be used because it has strong alkaline properties so that it can neutralize sputum and has a fairly good deproteination rate. In addition, NaOH does not interfere with DNA and does not degrade DNA so it is safe for use in the isolation process. However, in the application there are still many impurities or contaminations that are seen in the DNA band formed on electrophoresis. Therefore, other substances that have the same function as NaOH can be used as alternatives in the neutralizing and pre-deproteination stages (Jawetz, Melnick, Adelberg, 2007; Amin et al., 2011). There are other compounds that have properties such as NaOH and have a higher solubility, namely potassium hydroxide (KOH). Potassium hydroxide will neutralize sputum samples which are acidic and can help precipitate proteins and clean up various disturbing contaminations (Carlsson, Marek, and Keith, 1974).

Research Method

The population used in this study were all patients who examined their sputum samples in several Community Health Centers and hospitals in the city of Bandar Lampung. The determination of sample size in this study was determined based on the results of the Acid Resistant Basil examination. Where outpatients have been diagnosed with pulmonary tuberculosis on smear examination in accordance with the IUALTD (International Union Against Tuberculosis and Lung Disease) criteria. According to Kasjono and Yasril (2009) this type of research uses a sample size of 15 samples per group. The examination material used was tuberculosis patients' sputum. Sputum is obtained directly by isolating DNA from sputum samples after pre-deproteination first, then followed by PCR and electrophoresis (Gopi, Madhavan, Sharma, and Sahn, 2007).

Results and Discussion

This study used 15 samples of sputum that had been microscopically examined and diagnosed with pulmonary TB positive at several Community Health Centers and hospitals in Bandar Lampung City. Of the 15 sputum samples, there were 13 samples (87%) that were male and 2 samples (13%) were female, so there were 13 samples (87%) of productive age ie 15-50 years and 2 samples (13%) over 50 years old. The following are sputum sample characteristics data and the results of smear microscopic examination for each sputum sample conducted by the Public Health Center and hospital. Table 1 shows the percentages and results of smear microscopic diagnosis with Ziehl Neelsen's staining and sputum characteristics based on data obtained from several Community Health Centers and hospitals in Bandar Lampung City.

Table 1. Percentage Variations in Ziehl Neelsen's Staining Examination Results

Diagnosis Results	Number of Samples	Percentage
Positive 1	3	20%
Positive 2	8	53%
Positive 3	4	27%
Total	15	100%

The effect of using 1 N NaOH and 0.5 N KOH on the results of DNA isolation from sputum samples was seen in the thickness of the DNA band at electrophoresis. The thicker the DNA band seen on the electrophoresis results, the more DNA is isolated, the better the deproteination ability of the sputum sample by a substance capable of deprototeing, the purer the DNA produced (the absence of a smear). In this study it was seen that sputum samples deproteinated using 0.5 N KOH showed clearer DNA band results (see Figure 2). In addition, it was seen that the results of DNA isolation depreciated using 1N NaOH showed a long and clear smear compared to the results of isolation of sputum samples depreciated using 0.5 N KOH (see Figures 1 and 2). This can basically be seen directly, but in this study the results of electrophoresis were further processed using software, namely image J.

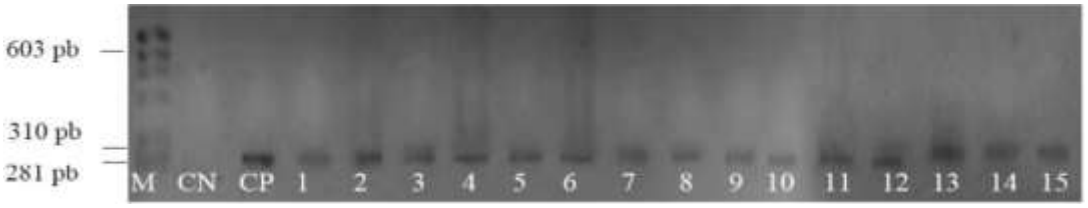

Figure 1. DNA Electrophoresis Results using NaOH (M: Marker, CN: Control Negative, CP: Control Positive)

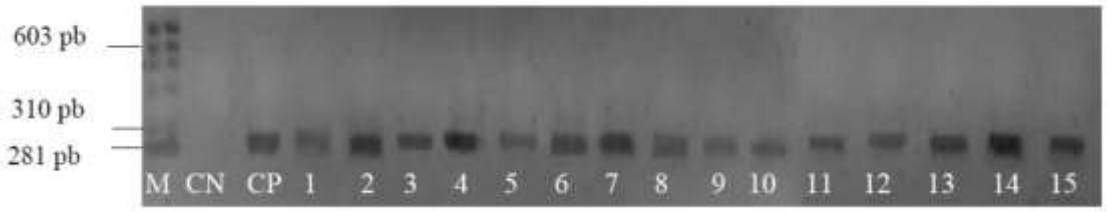

Figure 2. DNA Electrophoresis Results using KOH (M: Marker, CN: Control Negative, CP: Control Positive)

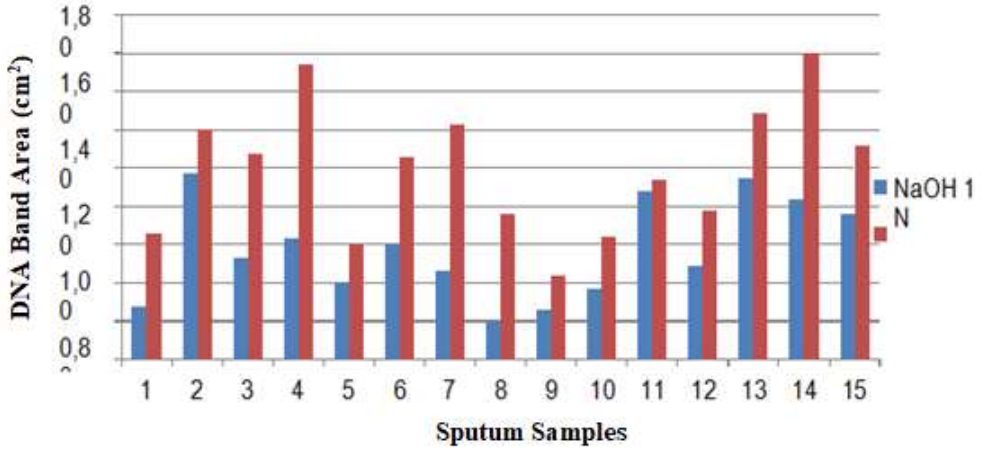

Figure 3. Measurement of Image J Results Based on Area of DNA Band from DNA Isolation Results using 1 N NaOH and 0.5 N KOH

Figure 3 shows the area of DNA bands treated using image J. Where the 15 samples that have been processed all show that the area of DNA band isolated from sputum samples deproteinated using 0.5 N KOH is greater than 1 N NaOH, this is in accordance with the seen visually in electrophoresis and the results of measurements using image J.

Sputum samples were pre-deproteinated using 1 N NaOH and 0.5 N KOH to remove proteins in the sputum, then proceed with the process of DNA isolation with the principle of lyse cell membranes or cell walls, extraction, precipitation, purification, and elution. This series of isolation processes is very long and continuous, mistakes at one stage will make the results of insulation is not good. Therefore, each phase of isolation must be considered, especially reagents used such as buffer lysis which serves to lyse cell membranes so that DNA comes out of cells, Proteinase K to degrade proteins, phenol-chloroform which functions to extract, NaOAC to remove other molecules besides DNA, cold ethanol for purification, and H2O free Rnase to preserve and dissolve DNA (Jawetz, Melnick, and Adelberg, 2007). After the DNA isolation process is carried out electrophoresis to see whether the results of DNA isolation that has been done produce DNA bands, if the isolated samples produce DNA bands on the electrophoresis then it can proceed to the next stage namely PCR to multiply or amplify the DNA results from the isolation process. Then after the isolation process is completed, the DNA from the isolation will be measured for purity and DNA concentration using a spectrophotometer.

This research was conducted to compare the use of two compounds that have similar characteristics, namely being able to dissolve, neutralize, and deproteinate sputum in the process of DNA isolation of Mycobacterium tuberculosis. The results of DNA isolation from

sputum samples deprototeed with 0.5 N KOH give better results compared to the results of sputum isolation depreciated with 1 N NaOH. Visually visible on the electrophoresis results from several sputum samples, KOH shows less smears than with NaOH. In the results of sample number electrophoresis 11 there was a smear in NaOH while in KOH there was no visible smear. As for samples number 2, 4, and 6 the NaOH looks long and clearly visible smears but on KOH only looks short smears. Sample number 1 was not seen in NaOH but in KOH there was a DNA band. For samples number 3, 5, 7, 8, 9, 10, 12, 13, 14 and 15 there were no visible smears, but DNA bands from sputum samples depreciated with 0.5 N KOH showed clearer results compared to DNA bands from sputum samples deproteinated with NaOH 1 N. The above can be caused by a long isolation process that starts from pre-deproteination of sputum samples, cell membrane or cell wall lysis, extraction, precipitation, purification, and elution have continuity in each process, so incorrect or inadequate treatment of each sample will be very influential when viewed on electrophoresis, whether there is a visible smear or no DNA band on the electrophoresis because the concentration of isolated DNA is too low. The better ability of 0.5 N KOH in deproteinating sputum samples for DNA isolation will make DNA purer and free from macromolecules such as proteins and RNA which are commonly called smears on electrophoresis (Ferreira and Rasband, 2010).

In addition to visualization seen on the DNA band in electrophoresis, the effectiveness of KOH in deproteinating sputum samples for DNA isolation is strengthened by measuring the area of the DNA band using a J image that measures the area of the DNA band based on the densitometry principle by looking at the thickness or density of the resulting DNA band (Ferreira and Rasband, 2010). Image J is widely used to measure DNA band thickness on electrophoresis or measure cell thickness as in the research of Ramadhani, Suvifan, and Lusiyanti (2013) looking at blast cell thickness and Aryantha, Mulyani, and Ariffudin (2008) DNA microsatellite research to find seed evaluation methods mushroom. The results of J image measurements were carried out 10 times in each band under study then the measurement results were averaged, the average results were processed using Excel to see the mean value, median, and standard deviation between NaOH 1 N and KOH 0.5 N.

The results of measurements with image J in Table 4.4 show that the process of neutralizing and deproteinating sputum samples using KOH 0.5 N has a larger area than NaOH 1 N. Image J measures the DNA bands produced on electrophoresis based on the area of each band DNA. The results of image J are comparable to the visualization of the DNA band seen on electrophoresis, the clearer the DNA band produced, the greater the area produced by image J. The results of image J measurements are processed using Excel to obtain the mean value, median, and standard deviation of each each test material, namely 1N NaOH and 0.5N KOH. There is a difference in the mean or mean value indicating that 0.5N KOH has clearer visualization results and a measurement of the image J is greater than 1N NaOH.

The use of KOH is more effective in deproteination compared to NaOH. The effectiveness of KOH in deproteinating sputum samples is because KOH has greater solubility than NaOH, so that KOH is able to hydrolyze hydrogen bonds to proteins faster and more effectively than NaOH. High solubility will make hydrogen bonds in KOH bind to hydrogen bonds in proteins to H2O or water (Taufiq, 2007).

Electrophoresis requires marker, positive control, and negative control to avoid errors in determining the base pair of DNA and prevent false positives or negatives. Markers serve as DNA markers to see the length of the DNA base pair, the base pair length for Mycobacterium tuberculosis is 281-310 base pairs (Ferrer, 1997). Positive controls are used to prevent false negative results due to errors in processing and as a reference for positive

DNA samples there are bands. Positive control can be used as a reference because it comes from pure culture of the bacterium Mycobacterium tuberculosis which will definitely show DNA bands that match the base pair of Mycobacterium tuberculosis DNA in the marker. Negative controls are used to prevent false positive results that occur due to errors in workmanship and to see whether there is contamination in the PCR process. Of the 15 sputum samples studied, all of these samples were in the base pair range corresponding to the marker and parallel to the positive control, and the absence of a band on the negative control showed that the PCR process was going well (Sameh, 2012).

This study provides information on the comparison of the use of KOH and NaOH in deproteination of sputum samples in DNA isolation of Mycobacterium tuberculosis that has not been previously reported. The information is expected to be able to add references and knowledge about the ability of KOH in depotreination for the isolation of Mycobacterium tuberculosis DNA. Potassium Hydroxide (KOH) with a higher depotreination ability can be used as a depoterination agent for sputum samples for DNA isolation of Mycobacterium tuberculosis to produce a clearer DNA band and no visible smears.

Conclusion

Visualization of DNA bands on electrophoresis using 0.5 N KOH is clearer than visualization of DNA bands on electrophoresis using NaOH 1 N. There is a mean difference between NaOH 1 N and KOH 0.5 N. The mean value of KOH 0.5 N is greater than NaOH 1 N with a mean difference of 0.43. Therefore, health workers are advised to be able to review and determine the diagnosis of pulmonary TB disease using a modified PCR method at the stage of DNA isolation using 0.5 N KOH so that the results of the DNA band look better and clearer.

Acknowledgements

We would like to say thanks a lot to the Lembaga Pengelola Dana Pendidikan (LPDP) Indonesian endowment fund for education, Finance ministry of Indonesia for supporting this research.

References

Amin, I., Muhammad, I., Zunaira. A., Muhammad, S., Samia, A., and Abrar, H. (2011). PCR Could Be A Methode Of Choice For Identification Of Both Pulmonary And Extra-Pulmonary Tuberculosis. *BMC Research Notes*, 4(2).

Aryantha, I. N. P., Mulyani, Y., and Ariffudin, R. (2008). Penanda Molekul DNA Mikrosatelit untuk Karakterisasi Bibit jamur Kuping (*Auricularia polytricha* [Month.] Sacc). *Jurnal Matematikan dan Sains*, 13(*1*).

Berger, H. W. and Meiia, E. (1973). Tuberculous Pleurisy. *Chest,* 63: 88–92.

Carlsson, J., Marek, P. J., and Keith, B. (1974). A Convenient Spectrophotometric Assay For The Determination Of L-Erghothioneine In Blood. *Biochem. J*, 139: 239 – 240.

Fatchiyah, A. (2011). *Biologi Molekuler Prinsip Dasar Analisis*. Jakarta: Erlangga.

Ferreira, T. A, Rasband, W. (2010). *The ImageJ User Guide Version 1.43*. Canada: McGill University.

Ferrer, J. (1997). Pleural tuberculosis. *Eur Respir J,* 10: 942–947.

Gomez, D. L., *et al*. (2012). Rapid DNA Extraction For Spesific Detection And Quantitation Of *Mycobacterium tuberculosis* DNA In Sputum Specimens Using Taqman Assay. *PMC*, 1: 3 – 4.

Gopi, A., Madhavan, S.M., Sharma, S. K., and Sahn, S. K. (2007). Diagnosis And Treatment Of Tuberculous Pleural Effusion In 2006. *Chest,* 131: 880–889.

Jawetz, Melnick, and Adelberg (2007) *Mikrobiologi Kedokteran. 23th ed*. Jakarta: EGC.

Kasjono, S. and Yasril (2009). *Teknik Sampling untuk Penelitian Kesehatan*. Yogyakarta: Graha Ilmu.

Kent, T. P. and Kubica, G. P. (1985). *Public Health Mycobacteriology: A Guide For The Level III Laboratory*. Atlanta : Center for Disease Control.

Kumar, P., Sen, M. K., Chauhan, D. S., Katoch, V. M., Singh, S., and Prasad, H. K. (2010). Assessment of the N-PCR assay in diagnosis of pleural tuberculosis: detection of *M.tuberculosis* in pleural fluid and sputum collected in tandem. *Plos One*, 5(4): 1 – 7.

Mirza, S., Restrepo, I. B., Mccormick, J. B., and Fisher-hoch, S. P. (2003). Diagnosis Of Tuberculosis lymphadenitis Using Polymerase Reaction On Peripheral Blood Mononuclear Cells. *Am J Trop Med Hyg*, 69(*5*): 461–465.

Mishra, A., Singhal, A., Chauhan, D. S., Katoch, V. M., and Srivastava, K. (2005). Direct Detection And Identification Of Mycobacterium tuberculosis And Mycobacterium bovis In Bovine Samples By A Novel Nested PCR Assay: Correlation With Conventional Techniques. *J Clin Microbiol*, 43: 5670–8.

Moon, J. W., Chang, Y. S., and Kim, S. K. (2005) The Clinical Utility Of Polymerase Chain Reaction For The Diagnosis Of Pleural Tuberculosis. *Clin Infect Dis*, 41: 660–666.

Nagesh, B. S., Sehgal, S., Jindal, S. K., and Arora, S. K. (2001). Evaluation of Polymerase Chain Reaction for Detection of Mycobacterium tuberculosis in Pleural Fluid. *Chest,* 119: 1737–1741.

Ramadhani, D., Suvifan, V. A., and Lusiyanti, Y. (2013). Otomatisasi Pendeteksian Sel *Blast* dan Sel Metafase dengan Perangkat Lunak Pengolahan Citra Sumber Terbuka OP Sel *Blast*. *Seminar Nasional Aplikasi Teknologi Informasi (SNATI)*, 1 – 13.

Seibert, A. F., Haynes, J., Middleton, R., and Bass, J. B. (1999). Tuberculous Pleural Effusion. Twenty-Year Experience. *Chest*, 99: 883–886.

Taufiq, A. (2007). *Biokimia Dasar*. Jakarta : Widya Utama.

Valde's, L., Pose, A., San, J. E., Martinez, V. J. M. (2003). Tuberculous pleural effusions. *Eur J Intern Med*, 14: 77–88.

World Health Organization (2013) *Health Topics Tuberculosis*. (Online). http://www.who.int/topics/tuberculosis/en/ . Accessed December 3, 2019.