EXAMINATION OF ACID-FAST BACILLI IN SPUTUM USING MODIFIED LIGHT MICROSCOPE WITH HOMEMADE LIGHT EMITTING DIODE ADDITIONAL ATTACHMENT

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Abstract

Typical clinical symptoms and chest X-ray is a marker of Tuberculosis (TB) sufferers. However, the diagnosis of TB in adults should be supported by microscopic examination. Currently, Bacilli microscopic examination of acid-fast bacilli (AFB) in sputum by Ziehl-Neelsen (ZN) coloring is the most widely used. However, for reasons of convenience, especially for laboratories with a considerable amount of smear samples, and due to higher sensitivity compared with ZN staining, the World Health Organization (WHO) has recommended the use of auramine-O-staining (fluorochrome staining), which is visualized by light emitting diode (LED) fluorescence microscopy. The aim of this study was to evaluate the performance of modified light microscope with homemade LED additional attachment for examination of AFB in sputum using auramine-O-staining method. We compared the sensitivity and specificity of 2 kinds of AFB in sputum methods: ZN and fluorochrome, using culture on Lowenstein-Jensen media as the gold standard. The results showed auramine-O-staining gives more proportion of positive findings (81%) compared to the ZN method (70%). These results demonstrated that the sensitivity of auramine-O-staining was higher than ZN, however it gives more potential false positive results than ZN. The sensitivity of auramine-O-staining in detecting AFB in sputum was 100% while the specificity was 88%.

Keywords: acid-fast bacilli, auramine, fluorescence microscope, light emitting diode (LED)

Introduction

Incidence of Tuberculosis (TB) cases has increased dramatically in recent decades throughout the world including in Indonesia. According to the Ministry of Health, the situation of TB in Indonesia is "unacceptable," with a number of new cases of about 539,000 and about 101,000 deaths per year.[1] World Health Organization (WHO)'s "Global tuberculosis control 2011" ranked Indonesia third in a list of 22 countries with the largest number of incident cases in 2010 after India and China.[2]

Typical clinical symptoms and chest X-ray is a marker of TB sufferers. However, the diagnosis of TB in adults should be supported by microscopic examination. Currently, sputum examination for acid-fast bacilli (AFB) by Ziehl-Neelsen (ZN) is the most widely used, especially in developing countries, including in Indonesia. Examination by ZN microscopy has a high specificity; however sensitivity is only about 50-80% and will decrease significantly until it reaches 20% in case of co-infection with HIV.[3,4]

Use of fluorescence microscopy is known to increase the sensitivity of 10% higher compared with ZN microscopy methods.[4] In fluorochrome staining, bacilli stained in stark contrast compared to the background as well as on examination under the microscope it does not require a magnification up to1000x. This can speed up the time of observation under a microscope and useful in the laboratory with the lots number of sample.[5] However, the use of fluorescence microscope in developing countries is not applicable due to high cost.

Light emitting diode (LED) fluorescence microscope is a diagnostic tool that is currently used as a replacement for conventional fluorescence microscope. This tool uses simple and low cost technology, but has the same quality as fluorescence microscopy. Light sources in these devices using LED, which has many advantages such as small size, the use of electrical energy with only a small power (20-100mA), and can be used with low voltage (2-5V).[6,7]

LED fluorescence microscope was reported to have performance equivalent to conventional fluorescence

microscope and does not require a dark room in operation. Sensitivity and specificity of the tool were reported to reach 84.7% and 98.9% compared to the gold standard (culture).[8] In another study, this tool has a sensitivity of 4% higher compared with ZN microscopy methods.[9] Meanwhile, according to the WHO study, this instrument has a sensitivity and specificity of respectively 84% and 98% compared to the gold standard (culture).[3] In the same report is known that sensitivity of the tool is 6% higher than that of ZN microscopy method and 5% higher compared with the conventional fluorescence microscope.

Currently LED microscope products have been widely available commercially. In addition, several light microscopy adapters for fluorescence observation, such as Paralens™ and Fraen After®, has also been available on the market. This study aims to see the performance (sensitivity and specificity) of light microscope that added non-commercial (homemade) LED additional attachment for AFB examination stained by auramine-O. The benefit to be obtained from this study is to increase the capacity of the laboratory in adopting a fluorescence staining (auramine) method.

Methods

Preparation of the microscope. The equipment was prepared for this study consisted of light microscopy, the excitation and emission filters, beam splitters, LED lights, and the voltage source adapter. Chart tool can be seen in Figure 1.

Filter made by printing the colors on A4 Yasica® transparent plastic using the HP Laser Jet 2605 printer. The print was then cut and pasted on acrylic. The colors composition of Red (R), Green (G), Blue (B) in the wavelength of filters that used in this study was 450 nm for excitation filter and 610 nm for emission filter. Excitation and emission filters were used in this study can be seen in Figure 2.

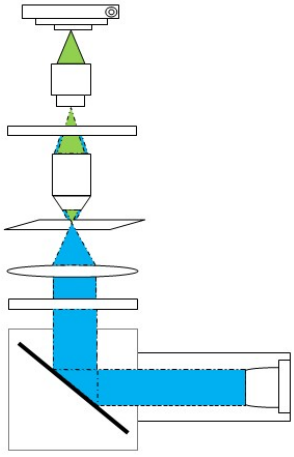

Figure 1. Scheme Tools Used: (a) LED, (b) Beam Splitter, (c) Excitation Filter, (d) Condenser, (e) Sample, (f) ObjectiveLens, (g) Emission Filter, (h) Eyepiece, (i) Camera

The LED light is a 3 watt high power LED with specifications: Luminous flux 30-160 LM, forward voltage 2.1-3.8 V and forward current 350 mA. LEDs is put in front of the beam splitter, under the microscope condenser. Settings tool on the light microscope can be seen in Figure 3.

Sputum examination. The total samples were 30 sputum from 30 suspected TB patients, which were sent to the Clinical Microbiology Laboratory, Faculty of Medicine University of Indonesia. Sputum specimens collected in wide mouthed sputum pots with screw cap, which was provided by the laboratory. Specimens that were not directly observed, stored in the 4 ^{o}C refrigerator.

Table 1. Conversion Value of Wavelenght to RGB

λ Filter*	R	G	B
Excitation (450 nm)	0	51	255
Emission (610 nm)	255	137	0

* Excitation wave length of auramine is 450-520 nm & emission wave length is 512 nm[10]. R: red, G: green, B: blue

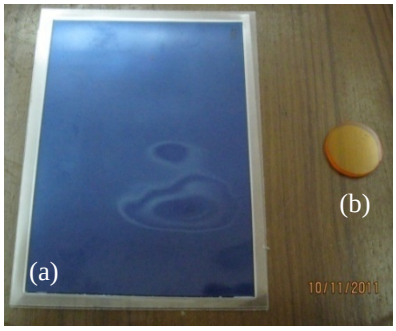

Figure 2. (a) Excitation Filter and (b) Emission Filter

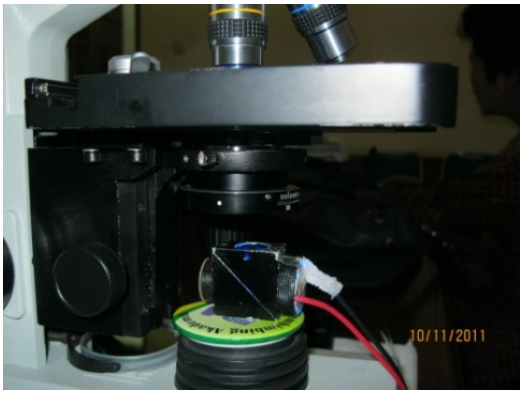

Figure 3. Settings Tool on Light Microscope

Preparations were conducted by smearing a single specimen on 4 object glasses that have been labeled with the laboratory code number. After air dried, smears were fixed over an open fire for 3-4 seconds. Smears that were not immediately stained, were stored in containers of preparations at room temperature.[5]

ZN staining was done by pouring a solution of 0.3% carbolfuchsin on the entire surface of the preparations, and then it was heated over an open fire until the smoke came out but not to boil or dry for 5 minutes. The stain is then poured out and the excess dye was removed and washed with running water. A solution of 3% acid alcohol (hydrochloric acid-ethanol) was poured on the smear for 2 seconds and directly washed with running water to remove excess solution. 0.1% methylene blue solution was poured to cover the entire surface, left it for 2 minutes then the solution was removed and washed with running water.[5]

Auramine-O staining was carried out by immersing the sputum smear in auramine solution (Merck), left it for 15 minutes then washed with chlorine-free water or distilled water and dried. The smear is then immersed in acid alcohol, left it for 2 minutes, washed with distilled water and dried. After that the smear was immersed in 0.5% potassium permanganate, left it for 2 minutes, washed it with distilled water and dried.[5]

The stains were observed under microscope at 1000x magnification. The amount of AFB was reported in accordance with the IUATLD scale (International Union Against Tuberculosis and Lung Diseases) in Table 2.

Culture method as a gold standard. Culture was done by planting sputum on Lowenstein Jensen medium after sputum homogenization process by Kubica. The culture was incubated at 37 °C and the growth was observed every week until 6 weeks.[11]

Table 2. IUTLD Scale[1]

Readings under the microscope *	Reporting the results
Bacilli is not found in the 100 field of view	Negative
1–9 Bacilli in 100 fields of view	Note the amount of Bacilli
10-99 Bacilli in 100 fields of view	+1
1–10 Bacilli in 1 fields of view	+2
>10 Bacilli in 1 fields of view	+3

* In auramine staining method, the reading done with 250x magnification

Results and Discussion

The results of AFB examination by auramine-O staining conducted in this study can be seen in Figure 4. Figure 4(a) was taken with an auto mode without using a flash. The resolution of the object is quite good, the bacilli looks quite obvious, but it is still difficult to distinguish between the bacilli with other fluorescent objects or background. Figure 4(b) produced by increasing contrast and reducing the light using the software 'Image Analyzer' to create color contrast among objects in the background. Based on both results, it can be concluded that the equipment used in this study captures the image of the fluorescence better when directly connected to computer in real time with image processing software.

The results of AFB examination stained by ZN and auramine-O-staining methods can be seen in Table 3.

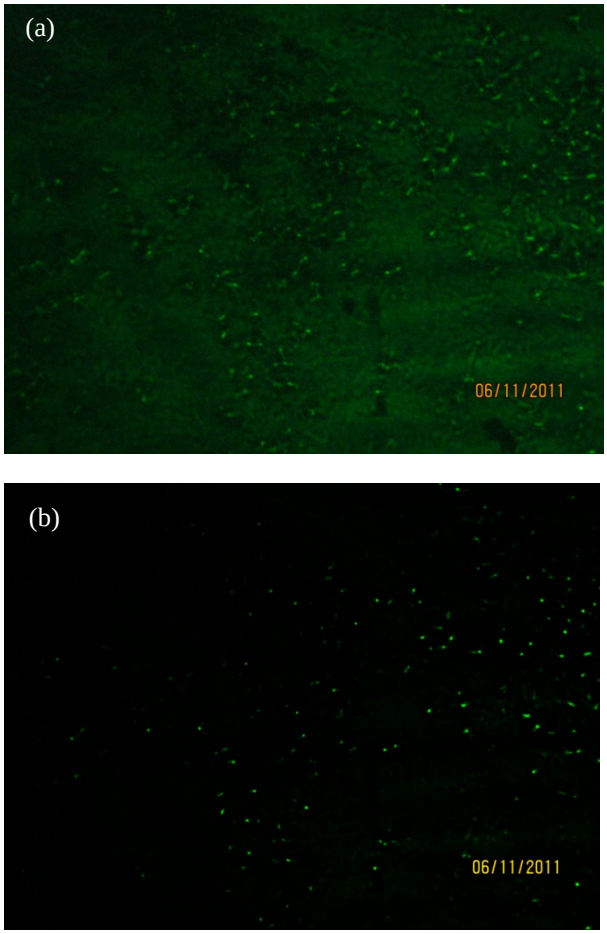

Figure 4. Sputum Smear were Observed Using A Modified Light Microscope in This Study: (a) The Original Image Taken with the Canon Powershot A3000 IS Camera, (b) Images Generated from the Reduction in Background Intensity

Table 3. The Results of AFB Examination by ZN Method Compared to Auramine-O-Stainiong Methods

Staining Results	Ziehl-Neelsen	Auramine Method
Negative	10 (33%)	7 (23%)
Positive	20 (67%)	23 (77%)
Total	30 (100%)	30 (100%)

Table 4. Staining of Smear Compared to Culture Examination

Culture (n)	Ziehl-Neelsen (n)	Auramine (n)
Negative (8)	(10)	(7)
Positive (22)	(20)	(23)
Total (30)	(30)	(30)

Table 5. Sensitivity and Specificity Values of Auramine and ZN Methods*

	Ziehl-Neelsen	Auramine
Sensitivity	91%	100%
Specificity	100%	88%

*Compared toculture, n= 30

Table 3 shows that the AFB examination with auramine-O method gives the proportion of smear-positive findings (77%) compared to the ZN method (67%). These results demonstrate that the sensitivity of the auramine-O method is predicted higher than ZN. It also indicates a great potential for false positives.

In some studies, auramine-O method was known to have high sensitivity and specificity, however, this method also has the great potential for false positive.[12,13] The high false positive values due to the visible fluorescence can be derived from tissue nonspecific, debris cell, dead bacilli, or even from inorganic material.[12] The results of AFB examination compared to the culture can be seen in Table 4.

Table 5 shows that ZN method has sensitivity that is not as high specificity. These results are in accordance with the study by Catanzaroetal (2000)[14] and Van Cleffetal (2003).[15] This occur because the number of bacilli in the sputum microscopically visible when sputum containing at least 10,000 Bacilli/mL.[4]

Conclusion

The modified light microscope in this study can be used to capture fluorescence images of the stained smear with auramine-O method. To increase the contrast between the bacilli and the background is recommended to connect the equipment to the image processing software.

The sensitivity and specificity of AFB examination using auramine-O method in this study were 100% and 88%. The sensitivity was higher than WHO study (84%), however the specificity was lower. The low value of specificity must be addressed among others by extending the experience of doing the proper staining and interpretation.

Acknowledgements

The research was funded through the Community Service Grant, University of Indonesia with number of contract: 1248/H2.R12/PPM.01.Pengabdian Pada Masyarakat/2011. We are also grateful to Drs. Kusmardi, MS and Mr. Trisno Budi Purnomo from the Department of Anatomical Pathology, Faculty of Medicine University of Indonesia for their help and suggestions that have been delivered.

References

1. Departemen Kesehatan Republik Indonesia. *Pedoman nasional penanggulangan tuberkulosis*. Jakarta: Depkes; 2007.
2. World Health Organization. *WHO health report: global tuberculosis control.* (internet) [cited in 2011]. Available from: http://www.who.int/tb/publications/global_report/2011/gtbr11_main.pdf.
3. World Health Organization. *Policy statement: fluorescent light emitting diode (LED) microscopy for diagnosis of tuberculosis*. Geneva: World Health Organization, 2010 March.
4. Amstrong D. LED-based fluoroscopy and the paralens system: illuminating the future of TB diagnostics. *Tuberculosis.* 2009; 7:17-18.
5. Karuniawati A, Risdiyani E, Nilawati S, Prawoto, Rosana Y, Alisyahbana B, Parwati I , Melia W, Sudiro TM. Perbandingan Tan Thiam Hok, ZiehlNeelsen dan fluorokrom sebagai metode pewarnaan basil tahan asam untuk pemeriksaan mikroskopik sputum. *Makara Kesehatan*. 2005; 9(1): 29-33.
6. Menn N. *Practical optics*. New York: Elsevier, 2004.
7. Feather JW, Ellis DJ, Leslie G. A portable reflectometer for the rapid quantification of cutaneous haemoglobin and melanin. *Phys Med Biol*. 1988; 33(6):711–722.
8. Marais BJ, Brittle W, Painczyk K, Hesseling AC, Beyers N, Wasserman E, van Soolingen D, and Warren RM. Use of light-emitting diode fluorescence microscopy to detect acid-fast bacilli in sputum. *Clinical Infectious Diseases*. 2008; 47:203–207.
9. Everett CK, Cattamanchi A, Davis JL, Worodria W, Den Boon S, Andama A, Yoo SD, Joloba M, Hopewell PC, Huang L. Performance of LED

fluorescence microscopy for the diagnosis of pulmonary tuberculosis: Preliminary results from the uganda national reference laboratory. *Am J RespirCrit Care Med*. 2010; 18:A1769.

10. Bird BR & Madison BM. Use of fluorochrome staining for detecting acid-fast Mycobacteria. *Department of Health and Human Services, Centers for Disease Control and Prevention*. 2000 December.
11. Koneman EW, Allen SD, Janda WM, Schreckenberg PC, Winn Jr WC, editors. *Color atlas and textbook of diagnostic microbiology*. Philadelphia: JB Lippincott Company; 1992.
12. Fraen Corporation. Detection of Auramine O stained Mycobacterium tuberculosis by means of transmitted light LED fluorescence – a new approach. (internet) [cited November 10th, 2011]. Available from: *http://www.fraensrl.com/images/Fraen_Microscope_tuberculosis.pdf*
13. McCarter YS, Robinson A. Detection of acid-fast bacilli in concentrated primary specimen smears stained with rhodamine-auramine at room temperature and at 37 degrees C. *J. Clin. Microbiol*. 1994; 32(10):2487-2489.
14. Catanzaro A, Perry S, Clarridge JE, Dunbar S, Goodnight-White S, LoBue PA, et. al. The role of clinical suspicion in evaluating a new diagnostic test for active tuberculosis. *JAMA*. 2000; 283(5):639-645.
15. Van Cleff MR, Kivihya-Ndugga L, Githui W, Nganga L, Odhiambo J, Klatser PR. A comprehensive study on the efficiency of the routine pulmonary tuberculosis diagnostic process in Nairobi. *Int J Tuberc Lung Dis.* 2003; 7:186-189.