

Comparison of Light Emitting Diode Fluorescent and Ziehl-Neelsen Staining Techniques with Culture for Diagnosis of Pulmonary Tuberculosis

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ABSTRACT

The objective of this study is to assess the validity of light emitting diode fluorescent and Ziehl-Neelsen staining techniques compared to the culture method for diagnosis of pulmonary tuberculosis (TB). All 272 clinical sputum specimens were collected from suspected pulmonary TB patients and were conducted by 2 staining methods and TB culture. The validity and agreement of both staining techniques compared with the culture were calculated and analyzed using Cohen's kappa statistics. The study revealed that the fluorescent staining technique had validity and agreement higher than Ziehl-Neelsen staining techniques. Additionally, the combination of both staining techniques can slightly increase the validity and agreement of the tests. Therefore, both fluorescent and Ziehl-Neelsen staining techniques remained the routine use of pulmonary TB diagnosis.

บทคัดย่อ

การศึกษานี้มีวัตถุประสงค์เพื่อประเมินความถูกต้องในการวินิจฉัยวัณโรคปอดด้วยวิธีการย้อมด้วยเทคนิคฟลูออเรสเซนต์และเทคนิคซีล-เนลเซน เปรียบเทียบกับวิธีการเพาะเชื้อ ตัวอย่างเสมหะทางคลินิกจำนวน 272 ตัวอย่างจะถูกเก็บจากผู้ป่วยที่แพทย์สงสัยว่าเป็นวัณโรคปอด ตัวอย่างเสมหะที่ได้ถูกนำมาวินิจฉัยด้วยเทคนิคการย้อมทั้ง 2 เทคนิคและวิธีการเพาะเชื้อ ความถูกต้องและความสอดคล้องของเทคนิคการย้อมทั้ง 2 เทคนิคเปรียบเทียบกับวิธีการเพาะเชื้อถูกนำมาคำนวณและวิเคราะห์โดยใช้สถิติ สัมประสิทธิ์แคปปาของโคเฮน การศึกษาแสดงให้เห็นว่าการย้อมด้วยเทคนิคฟลูออเรสเซนต์มีความถูกต้องและความสอดคล้องสูงกว่าการย้อมด้วยเทคนิคซีล-เนลเซน นอกจากนี้เมื่อรวมทั้ง 2 เทคนิคการย้อมและวิเคราะห์พบว่าความถูกต้องและความสอดคล้องของเทคนิคเพิ่มขึ้นเล็กน้อย อย่างไรก็ตามการย้อมด้วยเทคนิคฟลูออเรสเซนต์และเทคนิคซีล-เนลเซนยังคงมีความจำเป็นในงานตรวจวินิจฉัยวัณโรคปอด

Keywords: Fluorescent Staining Technique, Ziehl-Neelsen Technique, Culture

คำสำคัญ: เทคนิคการย้อมฟลูออเรสเซนต์ เทคนิคการย้อม ซีล-เนลเซน การเพาะเชื้อ

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Introduction

Tuberculosis (TB) is an infectious disease that is important to public health worldwide caused by *Mycobacterium tuberculosis* (MTB). The current TB situation reported by World Health Organization (WHO) in 2018 was an estimated 1.3 million deaths among HIV-negative people and 300,000 deaths among HIV-positive people (World Health Organization [WHO], 2018).

The best estimate is that 10.0 million people developed TB disease, 5.8 million men, 3.2 million women and 1.0 million children. There were cases in all countries and age groups, but overall 90% were adults (aged ≥ 15 years) and two thirds were in eight countries, India (27%), China (9%), Indonesia (8%), the Philippines (6%), Pakistan (5%), Nigeria (4%), Bangladesh (4%) and South Africa (3%). These and 22 other countries in WHO's list of 30 high TB burden countries accounted for 87% of the world's cases. Only 6% of global cases were in the WHO European Region (3%) and WHO Region of the Americas (3%) (WHO, 2018). In Thailand, estimated TB burden in 2017, the mortality in TB patients without HIV was 9,300 cases and the mortality of TB patients with HIV was 2,900 cases. The incidence of TB patients with HIV was 108,000 cases, the incidence of TB patients with HIV only was 11,000 cases and the incidence of MDR/RR-TB was 3,900 cases (WHO, 2018).

The culture is the gold standard method for TB diagnosis in clinical laboratory. Despite its high sensitivity and specificity, it is impractical in laboratory because of associated cost, labor intensive procedure, time-consuming and specialized safety measures (Bureau of Tuberculosis, 2018; Neelu, 2017). Therefore, most routine laboratory diagnosis of TB, the direct sputum staining is done by two techniques, Ziehl-Neelsen (ZN) based light microscopy which is based on the property of *Mycobacterium* species to retain the primary stain after exposure to strong acid-alcohol, the term is called Acid Fast Bacilli (AFB), and Light-emitting diode fluorescence microscopy (LED-FM) (Bureau of Tuberculosis, 2018; Gurung et al., 2018).

In this study, we aimed to compare the validity of two staining techniques using ZN technique and LED-FM technique with the TB culture for diagnosis of pulmonary TB using direct clinical sputum specimens.

Objectives of the study

To compare the validity of Ziehl-Neelsen (ZN) technique and Light-emitting diode fluorescence microscopy (LED-FM) with the TB culture (The Bactec MGIT 960 machine with SD BIOLINE TB Ag MPT64) for diagnosis of pulmonary TB using direct clinical sputum specimens.

Methodology

This study is a diagnosis study design, a total of 272 sputum samples were collected from suspected pulmonary tuberculosis patients ordered by a clinician for TB culture and examined in Tuberculosis Laboratory Center at Department of Microbiology, Army Institute of Pathology (AIP), Bangkok during July 2017 to April 2018.

AIP TB Laboratory received the sputum samples and conducted by routine laboratory examined. All the sputum samples were recorded into study log book using their allocated laboratory numbers and processed in a bio-safety cabinet (BSC). Demographic data of patients, age and gender were recorded.

All 272 sputum samples were prepared for smear two slides, one for ZN technique using TB Stain Kit ZN (Becton, Dickinson and Company) and the other for LED-FM technique using TB Fluorescent Stain Kit M (Becton, Dickinson and Company). ZN smears were examined under 1000X magnification of light microscope at least 100 high power fields (HPF) (one length) and LED-FM smears were examined under 400X magnification of LED fluorescent microscope at least 40 fields (one length) and the both of staining techniques were examined approximately 5 minutes before record the result. The staining results were graded by WHO, International Union Against Tuberculosis and Lung Disease (IUATLD) guidelines (Table 1).

Table 1 IUATLD/WHO guidelines for reporting sputum smear results

IUATLD/WHO scale (1000xfield = HPF) Grades	ZN staining based bright field microscopy (1000x magnification: 1 length = 2cm = 100 HPF)	LED-FM staining based sputum smear microscopy (400x magnification: 1 length = 40 fields = 200 HPF)
Negative	Zero AFB/ 1 length	Zero AFB/ 1 length
Scanty	1-9 AFB/ 1 length or 100 HPF	1-19 AFB/ 1 length
1+ grade	10-99 AFB/ 1 length or 100 HPF	20-199 AFB/ 1 length
2+ grade	1-10 AFB/ 1 HPF on Average	5-50AFB/ 1 field on Average
3+ grade	>10 AFB/ 1 HPF on Average	>50AFB/ 1 field on Average

The culture of TB diagnosis as a gold standard method from sputum samples was conducted of the decontamination by N-acetyl-L-cysteine (NALC) and cultured in liquid medium with Bactec MGIT 960 machine (Becton, Dickinson and Company). All positive cultures were conducted to SD BIOLINE TB Ag MPT64 (Abbott) which is rapid immune-chromatographic identification test for *Mycobacterium tuberculosis* by the MPT64 Ag detection in liquid media followed WHO for mycobacteriology laboratory manual (WHO, 2014).

Data Analysis

The data were analyzed for the validity comprising sensitivity, specificity, efficiency, positive predictive value (PPV) and negative predictive value (NPV) by MedCalc statistics online software version 18.5 (MedCalc Software bvba, Ostend, Belgium). The agreement was analyzed by Cohen's kappa statistics.

Ethics Approval

Ethical approval for the study was obtained from Ethical Review Committee for Human Research, Faculty of Public Health, Mahidol University, COA. No. MUPH 2017-150 Protocol No.111/2560, 5 July 2017 and Institutional Review Board Royal Thai Army Medical Department, IRBRTA 696/2017 Protocol No. S039h/59_Exp, 25 May 2017.

Results

A total number of 272 sputum specimens were collected and diagnosed by using microscopy examination ZN and LED-FM staining techniques compared with the TB culture. Among the 272 sputum samples, total of 101 (37.13%) and 119 (43.75%) sputum samples were positive for the presence of AFB by ZN and LED-FM techniques compared with 131 (48.16%) were culture positive for TB, respectively (Table 2).

Gender distribution and age distribution are shown in Table 2. In the gender, this study presence majority in males group 53.19% (n=100) and the age group 21-40 years (59.36%, n=38) of TB positive patients confirmed by culture, in the part of ZN positive majority in the age group 61-80 years (45.24%, n=38) and LED-FM positive majority in the age group 21-80 years (50.00%, n=115).

Table 2 Sputum examination results by age and gender

Characteristics	Examination	ZN Technique		LED-FM technique		Culture		
		Positive	%	Positive	%	TB Positive	%	
Gender	Male	188	80	42.55	90	47.87	100	53.19
	Female	84	21	25.00	29	34.52	31	36.90
Age	0-20	25	0	0	2	8.00	1	4.00
	21-40	64	27	42.19	32	50.00	38	59.36
	41-60	82	35	42.68	41	50.00	48	58.54
	61-80	84	38	45.24	42	50.00	39	46.43
	>80	17	1	5.88	2	11.76	5	29.41
Total		272	101	37.13	119	43.75	131	48.16

Table 3 Comparison of ZN and LED-FM with TB culture: Number of cases

Examination		Culture (Gold standard method)	
		TB positive	TB Negative
ZN technique	Positive	92	9
	Negative	39	132
LED-FM technique	Positive	105	14
	Negative	26	127
Combined tests of ZN and LED-FM techniques	One positive of both	106	14
	Both negative	25	127

Table 4 Comparison of validity of ZN and LED-FM with the TB culture (Gold Standard Method)

Validity of tests compared with the TB culture	Examination Techniques		
	ZN	LED-FM	Combination of ZN and LED-FM
Sensitivity	70.23%	80.15%	80.92%
(95%CI)	(61.62%-77.90%)	(72.29%-86.61%)	(73.13%-87.25%)
Specificity	93.62%	90.07%	90.07%
(95%CI)	(88.23%-97.04%)	(83.90%-94.46%)	(83.90%-94.46%)
Positive Predictive Value (PPV)	91.09%	88.24%	88.33%
(95%CI)	(84.33%-95.10%)	(81.91%-92.55%)	(82.06%-92.61%)
Negative Predictive Value (NPV)	77.19%	83.01%	83.55%
(95%CI)	(72.17%-81.54%)	(77.51%-87.38%)	(78.05%-87.89%)
Efficiency of test	82.35%	85.29%	85.66%
(95%CI)	(77.29%-86.69%)	(80.52%-89.28%)	(80.92%-89.60%)
Kappa	0.64	0.70	0.71
	(good agreement)	(good agreement)	(good agreement)

Table 3 and Table 4 show the validity and efficiency of staining examination. The ZN technique showed sensitivity, specificity, PPV, NPV, efficiency and Kappa were 70.23%, 93.62%, 91.09%, 77.19%, 82.35% and 0.64, respectively compared with the TB culture. The LED-FM technique showed sensitivity, specificity, PPV, NPV, efficiency and Kappa were 80.15%, 90.07%, 88.24%, 83.01%, 85.29% and 0.70, respectively compared with the TB culture. The combination technique between ZN with LED-FM showed sensitivity, specificity, PPV, NPV, efficiency and Kappa were 80.92%, 90.07%, 88.33%, 83.55%, 85.66% and 0.71, respectively compared with the TB culture. These results indicated that the LED-FM technique had more validity and good agreement than the ZN technique.

Discussion and Conclusions

In this present study, during July 2017 to April 2018, a total of 272 sputum samples were collected from suspected pulmonary tuberculosis patients who visited at Phramongkutklao Hospital and a clinician ordered for TB culture. All sputum specimens were examined in Tuberculosis Laboratory Center at Department of Microbiology, Army Institute of Pathology (AIP), Bangkok.

Our data show that the smear positive cases with respect to gender was found out to be 47.87% for males and 34.52% for females. The proportion of male patients was higher than female patients similar to previous studies reported (Neelu et al., 2017; Makesh Kumar et al., 2014; Reza et al., 2013). In the age group, the difference is most marked in the three age groups 21-40, 41-60 and 61-80 which were 50.00% of smear positive cases. This differed from other studies which were most in age group 51-60 years (Neelu et al., 2017) or 15-24 years (Reza et al., 2013).

Our study reveal, A total sputum specimens were smear positive detected by ZN staining technique were found out to be 101 (37.13%), whereas, smear positive samples detected by LED-FM staining technique were found out to be 119 (43.75%). The results revealed that proportion of smear positive samples by LED-FM staining technique was higher than positive samples by ZN staining technique. These were similar to previous studies (Neelu et al., 2017; Makesh Kumar et al., 2014; Reza et al., 2013; Goyal et al., 2013; Lawrence et al., 2016). Combination of two staining techniques were revealed the higher of smear positive sputum specimens 120 (44.12%) caused from one sputum specimen which was smear positive by ZN staining technique while LED-FM staining technique was smear negative. This sputum specimen was scanty grading results which should be caused by the sputum specimen collection, smear process or expertise of the microscopy staff.

The comparison of microscopy staining technique with TB culture was revealed that the results of positive for TB culture and MTB identification confirmed were 131 (48.16%) and other positive culture cases were Non-Tuberculous Mycobacteria (NTM) 19 (6.99%). The validity and agreement in this study revealed that the validity and agreement (Kappa) of LED-FM staining technique was higher than ZN staining technique. The validity and agreement (Kappa) of the combination of two staining techniques was higher than both of LED-FM staining technique and ZN staining technique.

AFB smear microscopy using conventional light microscope still remains the mainstay for diagnosis and monitoring treatment of pulmonary TB patients as it is simple, inexpensive, widely applicable and highly specificity for TB in endemic countries. Although, sputum specimens with low quantity or low density of bacilli, the reliability of LED-FM staining technique is much greater than that of ZN staining technique and significantly increase the diagnostic value of the smear.

Disadvantage of AFB smear microscopy could not easily differentiate between MTB and NTM because both were member of mycobacteria and positive results stained by both staining techniques. In 2016, WHO policy guidance for new diagnostic test, the TB loop-mediated isothermal amplification (TB-LAMP) is TB rapid molecular test for diagnosis of pulmonary TB (Ghosh et al, 2017; Mishra et al., 2018; Moon et al., 2015). TB-LAMP (Loopamp™MTBC Detection Kit, Eiken Chemical Company Ltd., Japan) is a commercial kit for the detection of MTB directly from sputum specimen persons with signs and symptoms consistent with pulmonary TB (WHO, 2016). Therefore, this issue should be further studied.

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References

- Bureau of Tuberculosis. National Tuberculosis control Programme Guidelines, Thailand 2018. Bangkok: Aksorn Graphic and Design; 2018
- Ghosh PK, Chakraborty B, Maiti PK, Ray R. Comparative evaluation of loop-mediated isothermal amplification and conventional methods to diagnose extrapulmonary tuberculosis. *Ann Trop Med Public Health* 2017; 10(1): 160-4.
- Goyal R, Kumar A. A Comparison of Ziehl-Neelsen Staining and Fluorescent Microscopy for Diagnosis of Pulmonary Tuberculosis. *IOSR Journal of Dental and Medical Sciences (IOSR-JDMS)* 2013; 8(5): 05-08.
- Gurung R, Shrestha R, Poudyal N, Bhattacharya SK. Ziehl Neelsen vs. Auramine staining technique for detection of acid fast bacilli. *JBPKIHS* 2018; 1(1): 59-66.
- Lawrence, Debbarna M, Baveja CP, Kumar S, Khanna A, Sapriina J. Comparative Evaluation of Fluorescent Staining with Ziehl-Neelsen and Kinyoun Staining in the Diagnosis of Clinically Suspected Cases of Pulmonary Tuberculosis. *International Journal of Contemporary Medical Research* 2016; 3(7): 1970-4.
- Makeshkumar V, Madhavan R, Narayanan S. Prevalence of drug resistance in *Mycobacterium tuberculosis* in a teaching hospital of Kanchipuram district, Tamil Nadu, South India. *Am J Microbiol Res* 2014; 2: 35-40.
- Mishra B, Hallur V, Behera B, Preetam C, Mishra PN, Turuk J, et al. Evaluation of loop mediated isothermal amplification (LAMP) assay in the diagnosis of tubercular lymphadenitis: A pilot study. *Indian J Tuberculosis* 2018; 65: 76-9.
- Moon SH, Kim EJ, Tomono J, Miyamoto S, Mitarai S, Kim DW, et al. Detection of *Mycobacterium tuberculosis* complex in sputum specimens using a loop-mediated isothermal amplification (LAMP) assay in Korea. *JMM* 2015; 64: 1335-40.
- Neelu SP, Terin j, Himadri D, Kalyani M. Comparison of Ziehl-Neelsen, Kinyoun's and Fluorescent staining for detection of *Mycobacterium tuberculosis* in sputum samples before and after Petroff's concentration technique. *Asian J Pharm Clin Res* 2018; 11(4): 110-3.
- Noori MY, Ali F, Ali Z, Sharafat S. Comparison of Ziehl-Neelsen Based Light Microscopy with LED Fluorescent Microscopy for Tuberculosis Diagnosis: An Insight from a Limited Resource-High Burden Setting. *J Ayub Med Coll Abbottabad* 2017; 29(4): 577-9.
- Reza LW, Satyanarayana S, Enarson DA, Kumar AMV, Sagili K, Kumar S, et al. LED-Fluorescence Microscopy for Diagnosis of Pulmonary Tuberculosis under Programmatic Conditions in India. *PLoS ONE* 2013; 8(10): 1-5.
- World Health Organization. Global Tuberculosis Report 2018. Geneva: World Health Organization; 2018.
- World Health Organization. Mycobacteriology Laboratory Manual. Global Laboratory Initiative Advancing TB Diagnosis. Geneva Switzerland: World Health Organization; 2014.
- World Health Organization. The use of loop-mediated isothermal amplification (TB-LAMP) for the diagnosis of pulmonary tuberculosis: policy guidance. Geneva: World Health Organization; 2016.