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Research Article

Application of Self-prepared AFB Stain Kit in Forensic Medicine for Tuberculosis Diagnosis

Ruthairat Siriwattanarat[1*] and Yasudama Chaimad [2]

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ABSTRACT

Tuberculosis (TB), a highly contagious and severe disease, is caused by *Mycobacterium tuberculosis* of the Acid Fast Bacillus (AFB). During autopsies, bodies suspected of being infected with Tuberculosis will have lung biopsies taken and sent for examination at the forensic histopathology laboratory. The Ziehl-Neelsen (ZN) staining technique is used to diagnose TB from tissue slides; however, it is not commonly used in the workflow of forensic histopathology laboratories. Consequently, when tissue samples are dispatched for TB diagnosis, it requires analysis at an external agency. This incurs supplementary expenses and extends the time for result reporting. In this research, we prepared a self-prepared AFB stain kit for AFB stain following the ZN staining technique, but modified it without heating in the lung tissue slide staining step. Our objective was to compare the diagnostic efficacy of self-prepared AFB stain kit with the conventional AFB stain kit. The effectiveness of both AFB stain kits was assessed by three experts. All data were analyzed by covariance (ANCOVA). The *P* value is more than 0.05, which is considered not statistically significant for all various aspects that have been examined. This indicates that both AFB stain kits, without heating in the staining step, showed no difference in the results and can be used for TB diagnosis in forensic histopathology laboratories.

Keywords: *Mycobacterium tuberculosis*, Acid Fast Bacillus, forensic medicine

[1] Department of Science, Faculty of Science and Technology, Suansunandha Rajabhat University, Dusit, Bangkok 10300, Thailand

[2] Institute of Forensic Medicine at the Police General Hospital, Pathum Wan, Bangkok 10330, Thailand

*Corresponding author, email: ruthairat.si@ssru.ac.th

Introduction

Tuberculosis (TB) is a communicable disease that is a major cause of illness and one of the leading causes of death worldwide. According to estimates, over a quarter of the world's population has contracted TB, but most people do not go on to develop the disease, and some people do recover from the infection. About 90% of those who contract TB annually are adults, with men experiencing the disease at a higher rate than women. Although the disease most frequently affects the lungs (pulmonary TB), it can also affect other locations of body [1]. The number of new and relapsed patients receiving treatment in Thailand increased between 2014 and 2020. However, the COVID-19 pandemic's effects caused a decline in 2021–2022. Extrapulmonary TB patients made up 9–16% of all registrations, and since 2016, there has been a discernible increase in both new and relapse patient registrations. This is a result of the nation's centralized individual-based database being put into place, which started as an offline and online system in 2018. According to the WHO's Global Tuberculosis Report 2022, 71,488 patients in Thailand were registered for treatment in 2021, including those who had relapsed and new cases of TB. Compared to the predicted 103,000 instances of tuberculosis cases in the nation, this indicated a treatment coverage rate of 70%. There are 22,876 female patients and 48,612 male patients with tuberculosis, representing a 2:1 ratio [2]. For tuberculosis diagnosis, the ZN method is widely used in many developing countries. ZN stain from sputum has been preferred due to its relative simplicity, high specificity, low cost of reagents, and simple microscope requirements [3, 4]. However, the effectiveness of the ZN method varies widely, with its sensitivity ranging from 20% to 80% in sputum samples (a minimum of 104 bacilli per sputum slide is required to reach a diagnosis) [5, 6]. This variability is largely influenced by how carefully specimens are collected, how smears are prepared, and the thoroughness of inspecting stained smears. Consequently, a notable portion of cases go undiagnosed, particularly when this method is solely relied upon for diagnosis. Furthermore, financial limitations constitute a barrier, particularly in low- and middle-income nations [6]. Sometimes a person who has TB goes undiagnosed during their lifetime [7].

An autopsy is essential for methodically analyzing the body to ascertain the cause of death, providing insightful medical information, and obtaining proof for any necessary legal inquiries. However, due to possible exposure to a variety of infectious pathogens, such as HIV, hepatitis B and C viruses, and *M. tuberculosis*, autopsy professionals may face major health hazards. It has been reported that under the right circumstances, *M. tuberculosis* bacteria can live for weeks or months in a variety of environments. Live *M. tuberculosis* bacteria have been reported to remain in the tissues of deceased individuals for up to 36 days after autopsy [8]. After an autopsy, forensic histopathology confirms and refines macroscopic diagnoses, including the identification of any incidental pathologies discovered during the procedure [9].

When lung autopsy tissues are sent to the forensic histopathology laboratory at the Institute of Forensic Medicine (Police General Hospital in Bangkok, Thailand) for TB testing. Lung autopsy tissues are processed in the histology laboratory to produce microscopic slides, and are then stained using the ZN staining technique. However, this technique occurs infrequently in the workflow of forensic

histopathology because laboratories typically have limited quantities of commercial AFB stain kits due to financial limitations and dyes with a short storage period. Consequently, when a sample is sent for diagnostic testing during an autopsy, it must be analyzed by an external agency, incurring additional costs and delays in reporting the result. In the ZN staining technique, the tissue sample is first treated with the carbol fuchsin solution. Carbol fuchsin is a red dye used in combination with phenol and alcohol, facilitating good penetration into the cells. After the application of carbol fuchsin, the sample is briefly heated to enhance the depth of dye penetration and improve binding to the tubercle bacillus. Following heating, the samples are cooled and then treated with an acid-decolorizing solution to decolorize non-acid fast cells and structures. The AFB stain retains the carbol fuchsin dye, appearing bright red, while other cell structures lose color [10]. The final step in the staining procedure involves the application of the counterstain, methylene blue, which colors other cells and presents the background material in blue [11, 12]. However, vapor generated from phenol in carbol fuchsin solution can enter the body by breathing, skin contact, or ingestion, leading to both local and systemic toxicity. Locally, phenol may cause irritation of the nose, throat, eyes, and skin burns. Acute poisoning presents with symptoms such as an increased respiration rate, followed by a decreased respiration rate, decreased body temperature, cyanosis, muscular weakness, weak or occasionally rapid pulse, and coma. Death typically results from respiratory failure. Chronic exposure to phenol is characterized by systemic problems [13]. In this research, the objective is to compare the diagnostic efficacy of a self-prepared AFB stain kit with a conventional AFB stain kit in examining tissue samples obtained from autopsies to detect tubercle bacillus. Additionally, to mitigate the risk to the health of laboratory personnel from prolonged exposure to phenol vapors, we modified the staining procedure by reducing the ZN heating step in both AFB stain kits, following the Armed Forces Institute of Pathology Tissue Staining Method Manual [14]. If our test prove effective enough to detect tuberculosis, they will be able to reduce costs and accelerate the reporting of test results, allowing for more accurate and faster handling of infected corpses.

Materials and Methods

There are various steps to compare the effectiveness of both AFB stain kits in lung autopsy slides for TB diagnosis as follows.

Ethical approval

Ethical approval of research protocol was considered and certified as an exemption for research ethics (COA.1-106/2022) by the Suan Sunandha Rajabhat Ethics Committee.

Sample collection and preparation of lung tissue slides

Samples were collected from the lung lobes of one deceased person diagnosed with TB and one deceased person diagnosed with non-TB. These samples were obtained from the autopsy tissues at the Institute of Forensic Medicine (Police General Hospital in Bangkok, Thailand). To ensure an adequate number of slides for the staining evaluation process, lung tissue was cut to a size of 0.3x0.3x0.2 mm from

five different locations following the lung anatomy lobes (three lobes in the right lung and two lobes in the left lung) and preserved in 10% neutral buffered formalin for approximately 24–48 hours. Subsequently, the tissue underwent processing, including paraffin embedding, resulting in the production of 10 tissue blocks (5 blocks from TB tissue and 5 blocks from without TB). Each tissue block underwent paraffin sectioning processing, resulting in 24 tissue slides per block. In total, 240 tissue slides were obtained, consisting of 120 slides from lung tissue with TB and 120 slides from lung tissue non-TB.

Preparation of AFB stain kit (ZN method)

Conventional AFB stain kit: Use conventional AFB stain kit that have received certification standards from COA, Technical Data, and Material Safety Data Sheet (MSDS). The components of a conventional AFB stain kit are as follows: a 1% carbol fuchsin solution (total of 450 mL; containing 4.5 grams of basic fuchsin dye, 45 mL of 95% ethanol, and 20.25 grams of phenol crystals, mixed solution, and made up to 450 mL with distilled water); an 6% AFB decolorizer (acid alcohol solution) (total of 450 mL; containing 13.5 mL of hydrochloric acid and 436.5 mL of 95% ethanol); and a 0.3% methylene blue solution (total of 450 mL; containing 1.35 grams of methylene blue in 450 mL of distilled water).

Self-prepared AFB stain kit is prepared following the manual of histologic staining methods of the Armed Forces Institute of Pathology [14]. The components of a self-prepared AFB stain kit are as follows: a 1% carbol fuchsin solution (total of 500 mL; containing 5 grams of basic fuchsin dye, 50 mL of 100% ethanol, and 25 mL of phenol crystals (melted), mixed solution, and made up to 500 mL with distilled water); an 1% AFB decolorizer (total of 1,000 mL; containing 10 mL of hydrochloric acid and 990 mL of 70% ethanol); and a 0.14% methylene blue solution (total of 300 mL; prepare from methylene blue stock solution 500 mL (methylene blue 7 grams and 95 % ethanol 500 mL) to methylene blue working solution 300 mL (methylene blue stock solution 30 mL and distilled water 270 mL).

Staining of conventional AFB and self-prepared AFB stain kits

The 120 lung tissue slides with TB were divided into two sets: the first set, comprising 60 slides, was stained using a self-prepared AFB stain kit, while the second set was treated with a conventional AFB stain kit. Similarly, the slides non-TB were divided in the same manner. Both AFB stain kits follow the same staining process, except for the duration of staining with the carbol fuchsin solution. The staining steps include deparaffinization with xylene, rehydration with graded alcohol, staining with carbol fuchsin solution (30 minutes for the self-prepared AFB stain kit and 1 minute for the conventional AFB stain kit), decolorization with AFB decolorizer, washing with tap water, counterstaining with methylene blue for 1 minute, washing with tap water again, dehydration with graded alcohol, and finally clearing with xylene. Allow the slides to dry.

Evaluating the conventional AFB and self-prepared AFB stain kits

Evaluating the effectiveness of both AFB stain kits was done by three specialists from the Institute of Forensic Medicine (Police General Hospital in Bangkok, Thailand). Experts were selected to score slide tissue based on various criteria: 1) Performing duties as a doctor of Forensic Medicine for more than 5 years, 2) Graduates of forensic medicine, 3) Graduates of medicine, 4) Performing duties as a medical technician for more than 3 years, and 5) Medical technicians who have had specialized staining training. In this investigation, there are three specialists: two medical technicians and a forensic medicine professional with over five years of experience.

The 240 slides will be divided into four assessments. Each tissue slide will be selected through a blinded test [15, 16]. Each expert will receive 20 tissue slides. Therefore, in one evaluation round, all three experts will assess a total of 60 slides. The effectiveness of both staining kits was evaluated in various areas, as follows: the carbol fuchsin in tubercle bacillus staining, the methylene blue on the background staining, and reporting criteria for AFB slide after ZN staining. The interpretation result was compiled according to World Health Organization (WHO) criteria for AFB. The collected data was recorded and statistical analysis using SPSS version 29.0.2.0. The Analysis of Covariance (ANCOVA) was used for comparison between variables. If the p value is less than 0.05, it is considered statistically significant.

Results and Discussion

Carbol fuchsin solution in tubercle bacillus staining

The carbol fuchsin staining of tubercle bacillus in lung tissue slides was graded by three specialists on a scale of 0 to 5 (not found to be excellent). Analyzing the effects of carbol fuchsin in tubercle bacillus staining, univariate analysis showed that, the interaction between the lung tissue slides (with TB and non-TB) and frequency of rating of the staining kits are not significant ($p \geq 0.05$) for all the variables examined in the present study ($F = 0.000$, $\eta^2 partial = 0.000$), as shown in Table 1.

Table 1 The results of the rating scale and percentage scale of carbol fuchsin staining of tubercle bacillus in lung tissue slides and results of ANCOVA.

Slides	**Staining kits**	**Rating scale**							***p* value**
		0	**1**	**2**	**3**	**4**	**5**	**Total**	
TB	A	0	0	4	7	17	32	60	
		(0.0%)	(0.0%)	(6.7%)	(11.7%)	(28.3%)	(53.3%)	100%	
	B	0	0	3	7	11	39	60	
		(0.0%)	(0.0%)	(5%)	(11.7%)	(18.3%)	(65%)	100%	1.000
non-TB	A	59	0	0	1	0	0	60	
		98.3%	(0.0%)	(0.0%)	(1.67%)	(0.0%)	(0.0%)	100%	
	B	59	0	0	0	1	0	60	
		98.3%	(0.0%)	(0.0%)	(0.0%)	(1.67%)	(0.0%)	100%	
Total		118	0	7	15	29	71	240	
		49.2%	0%	2.9%	6.3%	12.1%	29.6%	100.0%	

A = self-prepared AFB kit, B = conventional AFB kit

0 = not found; 1 = improvement; 2 = fair; 3 = neutral; 4 = good; 5 = excellent

Based on the results from Table 1, the study of the staining of carbol fuchsin in TB tissues revealed that the carbol fuchsin solution in both AFB staining kits achieved the same scoring level, ranging from 3 to 5 points. However, the conventional AFB stain kit obtained a higher percentage score at levels 4 and 5 compared to the self-prepared AFB stain kit, although there was no statistical significance. The lower percentage score of the self-prepared AFB stain kit at every level can be attributed to the basic fuchsin dye used in preparing the carbol fuchsin solution, which is an old dye that has been unexpired, which reduces the coloring efficiency, and it takes much longer to stain than the conventional AFB stain kit. In addition, the concentration of other substances used in the self-prepared AFB stain kit may be different from the concentration of the commercial AFB kit, which affects the staining process. Here, both AFB stain kits detected false-positive results in non-TB tissue slides. A multitude of variables could be the cause of this. Including reagent contamination or technical mishandling [17].

Methylene blue on the background staining

The methylene blue staining on the background of lung tissue slides was graded by three specialists on a scale of 1 to 5 (improvement to excellent). A univariate study of the effects of methylene blue on background staining revealed that the interaction between the lung tissue slides showed that, the interaction between the lung tissue slides (with TB and without TB) and the frequency of rating of the

staining kits were not significant ($p > 0.05$) for all the variables examined in the present study (F = 0.000, η^2partial = 0.000), as shown in Table 2.

Table 2 The results of the percentage scale of methylene blue staining on the background of lung tissue slides and results of ANCOVA.

Slides	Staining kits	Rating scale							*p* value
		0	**1**	**2**	**3**	**4**	**5**	**Total**	
TB	A	0	0	0	15	45	60	TB	1.000
		(0.0%)	(0.0%)	(0.0%)	(25.0%)	(75.0%)	100%	100%	
	B	0	0	0	11	49	60	60	
		(0.0%)	(0.0%)	(0.0%)	(18.3%)	(81.7%)	100%	100%	
Without-TB	A	0	0	0	28	32	60	0	
		(0.0%)	(0.0%)	(0.0%)	(46.7%)	(53.3%)	100%	(0.0%)	
	B	0	0	0	20	40	60	0	
		(0.0%)	(0.0%)	(0.0%)	(33.3%)	(66.7%)	100%	(0.0%)	
Total		0	0	0	74	166	240	0	
		0 %	0 %	0 %	30.8 %	69.2 %	100.0%	0 %	

A = self-prepared AFB kit, B = conventional AFB kit

1 = improvement; 2 = fair; 3 = neutral; 4 = good; 5 = excellent

Based on the results from Table 2, the study of the staining of methylene blue on the background staining in TB tissues revealed that the methylene blue solution in both AFB staining kits achieved the same scoring level, ranging from 4 to 5 points. However, the conventional AFB stain kit obtained a higher percentage score at every level compared to the self-prepared AFB stain kit, although there was no statistical significance. The lower percentage scores of the self-prepared AFB batches at all levels occurred for the same reason as the carbol fuchsin staining: the use of old but unexpired methylene blue dye. Additionally, the self-prepared AFB stain kit's methylene solution has a lower concentration than the methylene blue of the commercial AFB stain kit and also contains 95% ethanol, which affects its decolorization properties [18]. Consequently, the staining of methylene blue in the self-prepared AFB stain kit appears lighter than that in the commercial AFB stain kit.

Reporting criteria for AFB slide after ZN staining

Lung tissue TB 120 slides were stained with both AFB stain kits. The TB tissues were directly observed under the microscope, and the maximum number of AFB per high-power field (original magnification, 400) was counted and reported in each slide by three specialists. Images were captured using Nikon's ECLIPSE E200; Nikon Instruments. The results were compiled according to WHO criteria for AFB, which quantified results as follows: Negative: No AFB per 100 fields, Doubtful negative: 1–2 AFB per 300 fields, Doubtful negative: 1–3 AFB per 100 fields, Doubtful positive: 4–9 AFB per 100

fields, 1+: 1–9 AFB per 10 fields, 2+: 1–9 AFB per field, 3+: 10 or more AFB per field [19, 20]. All collected data were recorded and analyzed. The results of ANCOVA were utilized to ascertain significant differences between both AFB kits. Univariate analysis indicated that the interaction between the conventional and self-prepared kits were not significant ($p > 0.05$) for all the variables examined in the present study ($F = 0.000$, η^2partial $= 0.000$), as shown in Table 3, and the AFB count per field is illustrated in Figure 1.

Table 3 The results of the percentage scale of AFB count in lung tissue with TB slides and results of ANCOVA.

Slide	**Staining kits**	**AFB count**					**Total**	***p* value**
		Negative	**1-9 AFB**	**1+**	**2+**	**3+**		
TB	A	0	0	6	29	25	60	1.000
		(0.0%)	(0.0%)	(10.0%)	(48.3%)	(41.7%)	(100.0%)	
	B	0	0	4	26	30	60	
		(0.0%)	(0.0%)	(6.7%)	(43.3%)	(50.0%)	(100.0%)	
	Total	0	0	10	55	55	120	
		(0.0%)	(0.0%)	(8.3%)	(45.8%)	(45.8%)	(100.0%)	

A = self-prepared AFB kit, B = conventional AFB kit

Based on the results from Table 3, the present TB tissue, consisting of 120 slides from the same TB lung organ but different locations, exhibited positive criteria for AFB slides in both AFB stain kits, graded from 1+ to 3+. TB tissue stained with both AFB stain kits did not yield negative results. Specifically, 10 (8.3%) slides showed 1–9 AFB per 10 fields (1+), 55 (45.8%) slides showed 1–9 AFB per field (2+), and 55 (45.8%) slides showed 10 or more AFB per field (3+). ZN staining of TB tissue samples indicates the association of tissue reaction with mycobacterial infection. However, ZN stain has a relatively low sensitivity for detecting tubercle bacillus, with a sensitivity range of 50% to 60% in cases of confirmed (bacillary) pulmonary TB and even lower sensitivity (< 30%) in HIV-positive or immunosuppressed patients and in children [21].

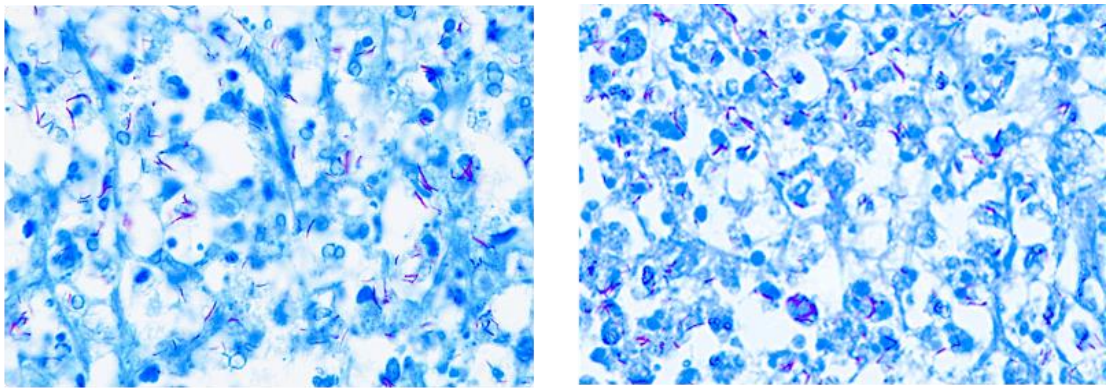

Figure 1 Staining with conventional AFB kit (left) and self-prepared AFB kit (right) in the same block tissue of lung with TB shown AFB count positive 3+ (10 or more AFB per field).

The AFB slide reporting criteria following ZN staining according to WHO guidelines, indicated equal effectiveness of both AFB staining kits in reporting outcomes. This parity in TB diagnosis reporting suffices for forensic medicine purposes, where reports are confined to either negative or positive results, devoid of patient treatment screening considerations. In Thailand, healthcare workers (HCWs) exhibited a TB incidence rate of 153 per 100,000 individuals (95% CI: 116 to 195), akin to the general population. Notably, a higher incidence was observed among HCWs in direct contact with TB patients and their specimens. Furthermore, a subgroup of newer HCWs with less than five years of experience displayed a notable TB incidence rate, with a significant proportion of asymptomatic HCWs diagnosed with TB [22]. A descriptive forensic post-mortem study investigating incidental tuberculosis-related deaths in sudden, unexpected, and violent community fatalities in Lusaka, Zambia, revealed a predominant occurrence of TB-related deaths in home or community settings. Forensic autopsies in community deaths unveiled previously undetected TB cases, with pulmonary disease observed in the majority of the cases. Pulmonary TB emerged as the primary cause of death in thirty-six cases and contributed to eleven others. Remarkably, none of the fifty-two TB decedents were suspected of having TB before death, and TB diagnoses were only apparent post-autopsy. All cases in the study were associated with low socioeconomic status and resided in densely populated areas of Lusaka. Given the prevalence of poor socioeconomic conditions and overcrowded living spaces in Lusaka, TB transmission remains a concern [23].

Conclusions

This research addresses issues encountered in reporting TB diagnosis results in forensic medicine at the Institute of Forensic Medicine, Division of Pathology (Police General Hospital, Thailand). A study compared a self-prepared AFB stain kit with a conventional AFB stain kit for TB diagnosis in forensic medicine followed by the ZN method modified without heating during the staining process. Data underwent ANCOVA analysis, with a p value more than 0.05 considered statistically not significant for all various aspects examined, as demonstrated. This indicates that the self-prepared kit performed as efficiently as the conventional AFB stain kits in forensic medicine. Therefore, employing self-prepared

AFB stain kits and heat-free processing in this study proved effective for forensic medicine reporting, not only reducing institutional costs but also facilitating more frequent TB diagnoses to enhance preventive measures among autopsy personnel.

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