Journal of Scientific Innovation in Medicine

Colored Cellophane Use in Sputum Smear Microscopic Examination Performance in Pulmonary Tuberculosis Diagnosis

RESEARCH

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ABSTRACT

Introduction: According to the World Health Organization (2020), staining and microscopy are important modalities in tuberculosis (TB) diagnosis, especially in rural areas, for screening, case-finding, and treatment evaluation.

Method: This pilot study compares the use of colored cellophane with standardized microscopy examination of stained sputum smear from seven patients with pulmonary TB in Surabaya Indonesia from March–June 2020. One-hundred forty images were captured and analyzed for contrast ratio and Acid-Fast bacilli count.

Results: The contrast ratio for each acid-fast bacillus and its adjacent pixels were significantly higher in yellow+blue filter group (3.24 ± 0.98) than in no filter group (2.52 ± 0.54). The contrast ratio for greyscaled images of yellow+blue filter group (3.53 ± 1.78) was significantly higher than no filter group (2.18 ± 0.83) ($p = 0.001$).

Conclusion: Colored cellophane increases contrast ratio and eases microscopic examination, and may potentially increase microscopic observation and detection capability.

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KEYWORDS:
Acid-fast staining; Microscopy; Tuberculosis; Sputum Smear; Cellophane

TO CITE THIS ARTICLE:
Edbert D, Mertaniasih NM, Endraswari PD, Koendhori EB. Colored Cellophane Use in Sputum Smear Microscopic Examination Performance in Pulmonary Tuberculosis Diagnosis. *Journal of Scientific Innovation in Medicine*. 2023; 6(1): 1, pp. 1–9. DOI: https://doi.org/10.29024/jsim.167

INTRODUCTION

Tuberculosis (TB) has become a world priority in health problems and one of the top ten causes of death by infection. In 2021, approximately 10.6 million people suffered from TB, 6million male, 3.4 million female, and 1.2 million children [1]. Multidrug-resistant TB (MDR-TB) is also a critical threat to public health. Between 2000–2019, 60 million lives were saved by accurate diagnosis and treatment [2]. In Indonesia, in 2019, 1,020,000 people suffered from TB, with an incidence rate as high as 391 per 100,000 population. TB prevalence in Indonesia is the second-highest after India [3, 4].

In its 2020 *Operational Handbook on Tuberculosis*[5], WHO listed microscopy as one of the primary bacteriological techniques in TB diagnosis and follow-up, especially in rural areas or remote country places; sub-district and community levels where molecular tests are unlikely to be performed. Microscopy remains the recommended modality to screen, as a primary case-finding method, and to evaluate treatment in resistant and susceptible TB cases. Limited resource facilities and peripheral rural areas in Indonesia preclude access to high end modalities such as nucleic acid amplification tests recommended by WHO such as Loop Amplification Method and Xpert MTB/RIF. The need for large-scale TB screening and medical professional shortage is increasing, but the technology is too advanced to be practical [6]. This emphasizes the need for more feasible optimization of the currently available diagnostic methods.

Many acid-fast modifications have been suggested to increase the effectiveness of microscopic observation, such as the use of oxidator agents [7–9], fluorescent microscopy [10], and bleach digestion [9] to shorten staining time and to ease the labor of microscope observers. Fluorescence microscopy has exceeded the sensitivity of common hot Ziehl Neelsen stain and cold Kinyoun-Gabbett-Tan Thiam Hok stain. Despite its excellent performance, fluorescence microscopy needs a special staining method, microscope, observation room, trained staff, and introduces specific hazards due to certain light waves and electron source [10].

Previous studies to improve acid-fast bacilli detection showed that centrifuged sputum and oxidized mycolic acid create a brown honeycomb pattern that is specific to acid-fast positive sputum [7, 8]. In 1973, Harada reported increased cell mass and higher contrast in pre-oxidized sputum slides by using permanganate [9]. Another study from 2012, used 30% peroxide to oxidize mycolic acid and to avoid the use of flame-heating claiming that the peroxide increases carbolic fuchsin penetration into the cell, thus enabling the observation of L-forms Mycobacteria [11].

Light wave modifications have also been made to increase optical contrast in microscopy, especially in fluorescence microscopy. Light is a complex phenomenon that can be modeled based on rays and wavefronts. These rays and waves can be diffracted as light passes through different substances, grids, or polarized films. In 1896, Julius H. Rheinberg introduced a paper to optically produce color contrast between an object and its background. Rheinberg's illumination was used to increase microscopic contrast, while giving hue to the microscope slides that is supposed to be monochromic [12]. It is a modification of dark-field microscopy, but this method uses translucent color filters instead of a black opaque annular ring. Common cellophane can be used for this method. This cellophane is placed under a microscope condenser and its size is adjusted to the size of the condenser opening [12–15]. A combination of blue and yellow filter using Rheinberg's method increased microscope contrast in 10-40x magnification [16].

In order to digitally process and identify microscopic images, a program algorithm must be set to separate the object from the background using threshold or histogram based techniques. [17] This conversion and the level number results depends on the dynamic range of the signal, which also depends on the illumination and RGB color space. Insufficient and non-uniform illumination decreases the intensity range for object and background, thus reducing the contrast ratio [18].

In rural area and limited resource settings, high-end modalities and additional chemicals might not be available. This pilot study aims to use common cellophane to intensify the contrast ratio in the observation and identification of bacteria in acid-fast staining–optically and digitally–in hope of increasing microscopic performance in limited-resource settings.

METHODS

ETHICAL STATEMENT

This research has been reviewed and approved by Ethical Committee of Dr. Soetomo Public Hospital (No. 0201/KEPK/V/2021). Consent from human subjects was acquired in written form and patient-related data are kept in confidentiality.

DATA COLLECTION

This pilot study is an experiment to compare the use of common colored cellophane and standardized microscopic examination of stained sputum slides from eight pulmonary TB patients_Surabaya Indonesia, from March-June 2020. The sputum samples were confirmed TB positive by Xpert MTB/RIF. Each sputum slide was duplicated and heat-fixed until ready to be stained.

STAINING

All slides were treated with Standard Ziehl-Neelsen using flame heated carbol-fuchsin 0.3%, HCl-alcohol 3%, and Levine's Methylene Blue.

OPTICAL OBSERVATION AND DIGITAL IMAGE ACQUISITION

A Nikon Eclipse E-100 LED was used to observe Ziehl-Neelsen stained slides. Colored pieces of cellophane were placed on the light source and inside the objective lens (Figure 1B). For each slide, ten separate oil immersion fields were captured using an Optilab Advanced + microscope camera mounted onto ocular lens fitting. The observation was divided into 3 groups, the no-filter, yellow filter, and yellow+blue filter group. A total of 240 images were obtained and saved

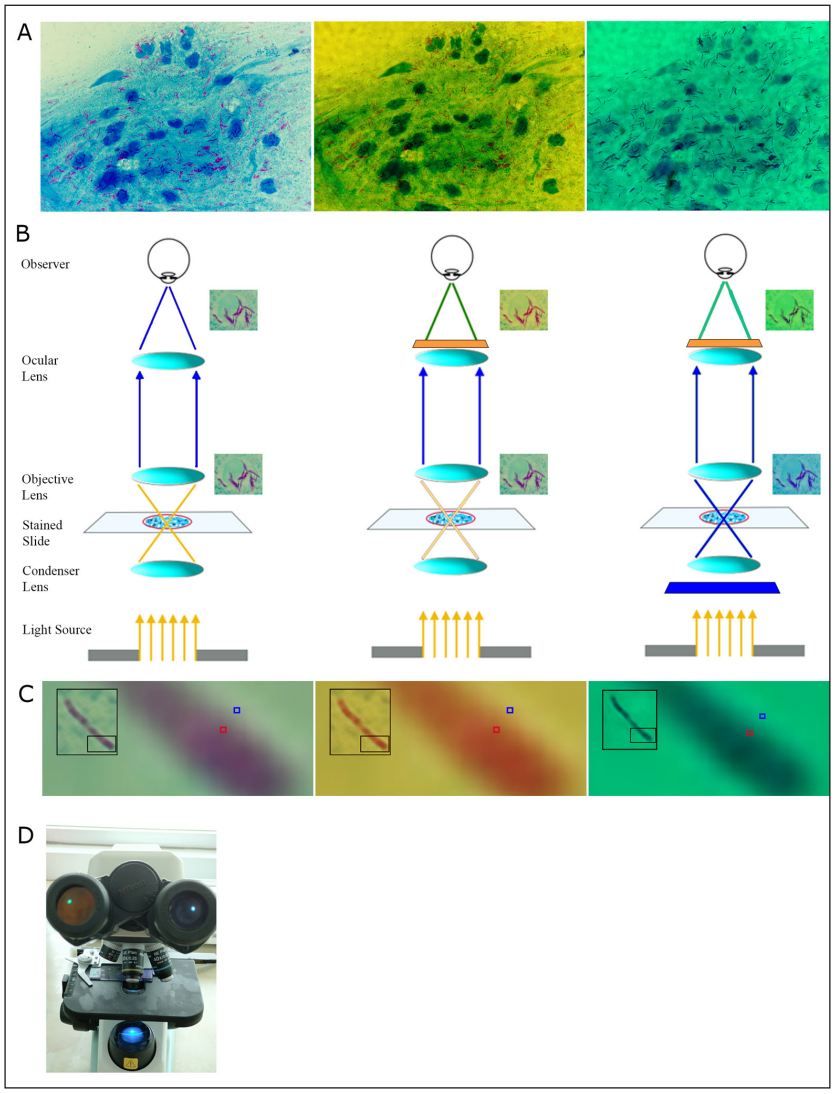

Figure 1 A. Comparison of the same microscopic field (objective 100x), the left image is the no-filter group, the middle is the yellow filter image, and the right image is the yellow+blue filter image. The use of filter removes background noise and non-acid-fast artifacts and reduces flare, thus creating sharper margin in each cell. **B.** The schematic diagram of filter placement and the images produced by the use of filter. The yellow filter group has red AFB and yellow background, similar to the use of light bulb microscope, but higher in luminosity, the yellow+blue filter group has black AFB and homogenous green background. **C.** The example of each AFB image produced by each filter placement and the point of sampling of adjacent pixels for contrast ratio analyses. **D.** The placement of filters for optical observation, the yellow filter is placed inside the camera lens adaptor for digital image acquisition.

in 2592 × 1944 JPEG format. Significant optical differences were only observed between the no-filter group and yellow+blue filter group. Therefore the images of the no-filter group and the yellow+blue filter group were converted digitally into grayscale mode and measured for contrast ratio.

DATA ANALYSIS

Contrast Ratio Comparison

A pair of RGB data of acid-fast bacilli pixels and its adjacent background was noted from each of the 240 acquired images and 160 grayscale-converted images to calculate a total of 400 contrast ratio data (Figure 1A). The acquired standard Red, Green, and Blue (sRGB) value was then converted into luminance and calculated into contrast ratio. The same method of contrast ratio calculation was performed on the grayscale converted images. The differences of contrast ratio between group was analyzed using Kruskal-Wallis test and post-hoc analysis was performed using Wilcoxon test. The differences of contrast ratio between the grayscaled images of the no-filter group and the yellow+blue filter group was analyzed using Wilcoxon test.

Acid Fast Bacilli Quantification

Observed acid-fast bacilli (AFB) was enumerated from each captured image. The maximum number of acid-fast bacilli counted by observer from each image in each slide is 50, which is 5x higher than maximum number of International Union Against Tuberculosis and Lung Diseases (IUATLD) scale interpretation. Mean of acid-fast bacilli count between no filter, yellow filter, and yellow+blue filter group was compared using Kruskal-Wallis test and post-hoc analysis was performed using Wilcoxon test. Microscope observation was performed independently by three different laboratory technicians and was recorded using the IUATLD scale. Inter-observer agreements and between group agreements were calculated using Cohen's Kappa.

RESULTS

Mean contrast ratio for each AFB and its adjacent pixel in yellow+blue filter group is 3.28 ± 1.64, higher than yellow filter group (2.10 ± 0.58) and no-filter group (2.56 ± 0.99) (p = 0.016; CI 95%). Post hoc analysis showed the contrast ratio between yellow+blue filter group and yellow filter group were significantly higher (p = 0.012; CI 95%). The contrast ratio in the filter groups were higher; yellow+blue filter vs. no-filter group (p = 0.012) and the yellow filter vs. no-filter group (p = 0.018). The contrast ratio of grayscaled pictures between yellow+blue filter group and no filter group were also significantly different (p = 0.012: CI 95%). AFB identification and enumeration per image in the filter groups are higher than no-filter group but did not differ significantly (p = 0.83; CI 95%) (Table 1). Post-hoc analysis showed significant differences in AFB enumeration using yellow+blue filter compared to no-filter group (p = 0.028; CI 95%) and yellow filter compared to no-filter group (p = 0.034; CI 95%). No differences between IUATLD

Table 1 Contrast ratio and acid fast-bacilli (AFB) count from 10 images per slide.

SPUTUM	ORIGINAL IMAGE CONTRAST RATIO			GRAYSCALED IMAGE CONTRAST RATIO		MEAN AFB COUNT/10 IMAGES		
	YELLOW+BLUE	YELLOW	NO FILTER	YELLOW+BLUE	NO FILTER	YELLOW+BLUE	YELLOW	NO FILTER
1	2.60	1.70	2.10	2.00	1.90	2.7	2.3	1.9
2	2.30	1.60	1.90	1.60	1.40	1.3	1.3	1.3
3	3.10	2.20	2.60	3.40	2.00	15.2	15.5	13.5
4	4.30	2.50	2.80	5.20	2.90	50.0	50.0	49.9
5	5.00	3.20	3.20	6.30	3.80	39.0	38.6	38.3
6	3.50	2.40	3.30	4.80	2.20	34.7	34.5	34.3
7	2.30	1.50	2.10	1.50	1.20	44.4	44.2	44.4
8	2.81	1.90	2.17	3.40	2.00	20.0	19.2	18.0
Mean ± SD	3.24 ± 0.98	2.13 ± 0.57	2.52 ± 0.54	3.53 ± 1.78	2.18 ± 0.83	25.9 ± 18.8	25.7 ± 18.8	25.2 ± 19
Median	2.96	2.05	2.39	3.40	2.00	N/A	N/A	N/A

scale between the three groups (p = 1.000; CI 95%). Percent of agreement of IUATLD scale between filter use and without filter use was 100% (Cohen's K = 1; CI 95%) (Table 2).

Table 2 International Union Against Tuberculosis and Lung Disease (IUATLD) scale in direct microscopic observation.

IUATLD SCALE			
SPUTUM	**YELLOW+BLUE**	**YELLOW**	**NO FILTER**
1	Scanty – 2+	Scanty – 2+	Scanty – 2+
2	Scanty – 1+	Scanty – 1+	Scanty – 1+
3	3+	3+	3+
4	3+	3+	3+
5	3+	3+	3+
6	3+	3+	3+
7	Scanty – 1+	Scanty – 1+	Scanty – 1+
8	3+	3+	3+

Between the digitally converted images (Figure 3), the contrast ratio for each AFB and its adjacent pixel in yellow+blue filter group is 3.5 ± 1.8, higher than the contrast ratio in no-filter group (2.6 ± 0.99) (p = 0.001; CI 95%). AFB identification and enumeration per image does not differ significantly among the three groups (p = 0.82; CI 95%).

DISCUSSION

The burden of eye strain and musculoskeletal problems have negatively affected microscope operators. A study in Teheran reported approximately 2.5 hours of microscopic work in a day have a strong positive correlation to visual fatigue. Visual fatigue, listed as dry eyes, burning eyes, headache, tearing of the eye, and drowsiness are the five most occurring complaints in high-burden microscope operators [19]. Increasing the contrast ratio by 1.29 fold will hypothetically shorten observation time, thus reducing operator burden. In this study, increasing the contrast ratio also resulted in higher AFB count per oil immersion field, thus increasing the identification capability, especially in scanty microscopic fields.

The potential to use image digitization will also contribute to less need for trained microscope operators and faster identification of AFB in digitally acquired images. In this research, the grayscaled images in the yellow+blue filter group have only two channels (the red channel is totally black), whereas in the grayscaled images in the no-filter group have three channels (the common red, green, and blue channels). This is another advantage for image processing algorithms to identify objects. The contrast ratio also increases 1.62 fold in the grayscaled images by using yellow+blue filter.

The microscope mounts a light-emitting diode (LED) as the light source to balance the color spectrum. The microscope has a more uniform light wave spectrum, compared to the common halogen lightbulb which emits more orange to yellow light waves [20]. The light intensity was higher than the light bulb microscope. This is necessary to reduce light wave bias for a research. Certain hue adjustment are needed for different types of light source, as there are still many health facilities in Indonesian rural area that still use light-bulb microscopy.

Stained sputum slides act as a light filter as light passes through the translucent body of a cell. The denser the structure is, the less light will pass, resulting in a darker image. Ziehl-Neelsen staining is used as a standard procedure to identify acid-fast bacteria. Direct heat to carbol fuchsin covered slides melt mycolic acid which has a melting point of 53–55.5°C and allows cationic carbol fuchsin to intercalate with anionic structures [8]. Through polarization of light, carbol fuchsin dye bends specific light waves with its unique refractive index, allowing only red light waves to pass [21]. Proteins that are denatured by acid and counter-stained structures stained with methylene blue allow only blue and green light waves to pass [22].

When this red light is blocked by a yellow filter, no red light wave passes through, causing the object to be perceived as black. The blue artifacts are visually made vague by an additional

blue filter, making all backgrounds appear relatively homogenously colored [22]. The additional yellow filter then filters any remaining red and violet light waves, thus creating a homogenous green background (Figure 1C and 1D).

The blue color filter used in this study reduced the brightness of each observation field. Black acid-fast bacteria relying on morphotype identification only might appear curious to common technicians' eyes. If applied, this method needs a proper period of adjustments to familiarize the differences in observation. Although Rheinberg's photomicrography had been used for various microscopic observations, there is no report regarding its use in the oil immersion field [13, 14]. We believe this is the first reported study using light wave modifications to observe stained bacteria.

Advantages of the filter used in this research are:

1) According to interviews of the microscope operators in this study, optical fatigue is reduced and the personal capacity of microscope operator might increase in reading acid-fast slides daily. This might result from the reduction of blue light waves and light flares
2) Higher contrast ratio enhances the ability of AFB detection if familiar with its morphotype, thus reducing time to read each slide (Figure 2A and 2B);
3) The ability to identify bacterial structures and formation in higher contrast and clearer margin, reduction of light diffraction, especially in superposition with human cells or non-acid-fast bacteria. This method might better distinguish bacterial morphology;
4) In case of imperfect decolorization, AFB are still visible although seems faint (Figure 2C). In preliminary tests, this method is also applicable to Fite's modification stain and Kinyoun-Gabbet-Tan Thiam Hok cold staining method.
5) Low cost of using cellophane as an optimization tool in microscopy.

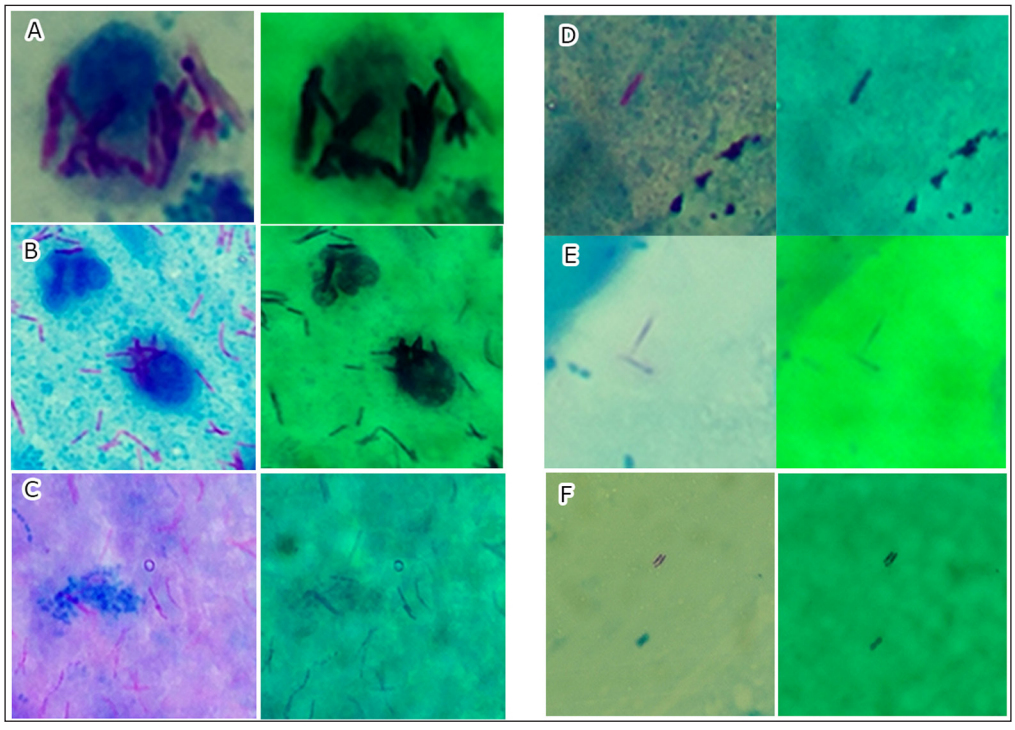

Figure 2 Advantages and disadvantages of filter use. **A:** AF components can be more visualized and can be used to observe AFB in thick and purulent specimens. **B:** Filter use can unveil "hidden AFB" inside human cells. **C:** In poor decolorization, AFB can still be visualized clearly. **D, E, F:** The same dark bluish-black color can appear from artifacts and non-acid fast bacteria.

Disadvantages of this filter use are:

1) Microscope operator must be familiarized with the technique and bacterial morphotype to reduce misreading (Figure 2E and 2F); Proper training in bacterial morphotype identification is needed. Thin artifacts like dye crystals can be misinterpreted as AFB if the observer lacks proper knowledge of morphotypes.
2) Filter use might reduce light intensity, thus increasing the need for an optimal light source with a light-emitting diode or white light bulb.
3) There might be tiny folds or cellophane defects that might interrupt microscopic reading.

In order to construct the digital image processing algorithm, images have to show significant contrast in grayscale mode (Figure 3). Specific objects can be identified using shape and color properties or, in this case, the difference of luminance or intensity [18]. With the use of cellophane, the contrast significantly increases and the background illumination is uniformly spread throughout the image. This important step will increase the accuracy of digital image processing algorithm.

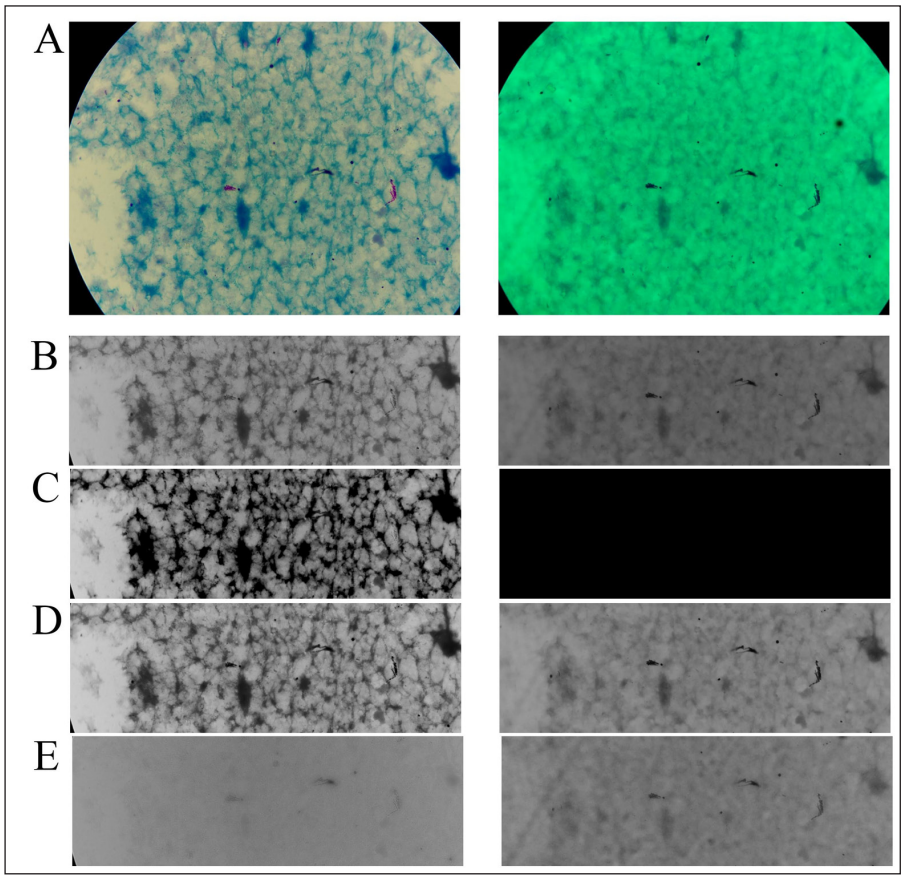

Figure 3 Comparison of digitally grayscaled images of the same observation field in no-filter group (left) and yellow-blue filter group (right). The original image is shown in A. The gray-scaled section is shown in B. The color channels; Red (C), Blue (D), and Green (E) showed significant differences. The use of filter significantly differentiate the object form background noice in the blue channel.

LIMITATIONS

This study is limited in sample size and microscope type. A larger-scale study is needed to increase accuracy and evaluate larger-scale application. Various clinical specimens and microscope types might improve the diagnostic value of this new method.

CONCLUSION

The use of common yellow and blue cellophane as a color filter is the best combination to intensify contrast ratio in acid-fast microscopy and eases AFB identification and enumeration, both optically and digitally.

ACKNOWLEDGEMENT

We thank Dr. Soetomo Academic Hospital for providing all necessary support in this research.

COMPETING INTERESTS

The authors have no competing interests to declare.

AUTHOR CONTRIBUTIONS

DE designed the study. DE, PDE, and EBK designed the methodology. DE performed the experiments. PDE and NMM supervised the experiments. DE and NMM analyzed the data. DE, PDE, and NMM wrote the paper with input from all authors.

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TO CITE THIS ARTICLE:
Edbert D, Mertaniasih NM, Endraswari PD, Koendhori EB. Colored Cellophane Use in Sputum Smear Microscopic Examination Performance in Pulmonary Tuberculosis Diagnosis. *Journal of Scientific Innovation in Medicine*. 2023; 6(1): 1, pp. 1–9. DOI: https://doi.org/10.29024/jsim.167

Submitted: 14 July 2022
Accepted: 25 October 2022
Published: 25 January 2023

Journal of Scientific Innovation in Medicine is a peer-reviewed open access journal published by Levy Library Press.