

## Added value of bleach for tuberculosis microscopy diagnostic in limited resources setting

Erike A. Suwarsono,<sup>1</sup>  
Siti Nur Aisyah Jauharoh<sup>2</sup>

<sup>1</sup>Microbiology Department, <sup>2</sup>Clinical Pathology Department, Faculty of Medicine, UIN Syarif Hidayatullah Jakarta, Indonesia

### Abstract

Indonesia is one of the high burden tuberculosis (TB) countries. The utilization of molecular diagnostic has been setting over the country, however there are still a lot of remote areas that only depend on microscopic smear. Bleach might be used to increase the value of microscopy diagnostic. The study aimed to evaluate the added value of 1% bleach to increase the positivity rate of TB compared to conventional stain. This study was conducted on 70 subjects who were suspected as TB from various primary care centers in West Java and Banten, Indonesia. The sputum was 2-3 mL, and divided into two parts, both of them were stained using Ziehl-Neelsen (ZN), however the first was only stained and the second added 1% bleach. Bleach was added in same volume with sputum and incubated for 10 minutes. After 10 minutes, the sputum was stained with ZN. The positivity rate for ZN only was 39 (55.7%) compared to 1% bleach was 63 (90%), and significantly different ( $P=0.000$ ) using Mann-Whitney U test. Bleach can be used as an alternative agent to improve the TB smear in setting that only depends on microscopy diagnostic.

### Introduction

Tuberculosis (TB) is still one of major health problem around the globe, especially in developing country, such as Indonesia. According to WHO, Indonesia is one of 22 high burden TB countries, with moderate-high prevalence of TB, TB-MDR and TB-HIV.<sup>1</sup> The Indonesian government already conducted a massive program in order to control TB infection, including set up a molecular diagnostic tool free of charges in many of health center. However, Indonesia is a big country with an archipelago geography, there are many of small islands and

remote area that still lacks of TB diagnostic's capability and only depends on microscopy. The Ziehl-Neelsen (ZN) staining, an over 100 years old diagnostic method, has become most used technique, although the sensitivity is variable.<sup>2</sup> There are many methods to increase the sensitivity of smear microscopy, including bleach. A previous study showed that bleach homogenisation and overnight sedimentation could increase the sensitivity, whereas another study found that centrifugation resulted in higher yields than sedimentation.<sup>3-5</sup> However centrifugation and sedimentation need electricity and more advanced equipment, that may not meet in primary health care centers or limited resources health centers. This study was conducted to evaluate the use of 1% bleach to be added before ZN stain in order to increase the positivity rate of smear microscopy compared to ZN stain only. In this study, we used 1% bleach without centrifugation or any other technique to concentrate the sputum, because we want to make the technique easier to implement in limited resource setting.

### Materials and Methods

In this study, 70 suspected new TB patients from several primary health cares in Bekasi and Tangerang Selatan region, Indonesia, were eligible. The sputum samples were collected after patient signed the informed consent during September-December 2018. The study was approved by the ethical committee of Faculty of medicine UIN Syarif Hidayatullah Jakarta (the number of ethical clearance 617/UN2.F1/ETIK/VII/2018). All laboratory work was conducted in the microbiology laboratory of the Faculty of Medicine, UIN Syarif Hidayatullah Jakarta.

The patient submitted sputum in 2-3 mL, and we divided the sputum into two parts. The first part for ZN stain and the second part for ZN added 1% bleach. The bleach added to the sputum in same value, and was shaken manually for less than a minute the closed tight the container, and incubated for 10 minutes in room temperature before stained by using ZN. We carried out the ZN technique as previously described.<sup>6</sup> We diluted 5.25% commercial bleach into 1% bleach and stored it in a dark bottle to avoid from direct sunlight. Each smear was examined by binocular microscopy (magnification x1000) on site by two different persons for validation. Each laboratory technician examined both

Correspondence: Erike A. Suwarsono, Faculty of Medicine, UIN Syarif Hidayatullah Jakarta. Jl. Kertamukti No. 5 Cirendeui, Ciputat Timur, Tangerang Selatan, Banten, 15412, Indonesia. Tel.: +62 (021) 74716718; Mobile phone: +62 8165426218. E-mail: erike.suwarsono@uinjkt.ac.id

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smears consecutively and validated each other blindly. The result was positive if there were more than one acid fast in 100 observations field be founded. The result was not categorized by IULTD's score (1/2/3), however it was assigned qualitatively to positive or negative smear. For the internal quality, 100% positive and 10% of negative smear from bleach and non-bleach group were blindly reexamined by clinical microbiologist.

We compared the positivity rate between each group and analysed it statistically with Mann Whitney-U test to test statistical difference as the sample was paired specimen. We also made description for the characteristic's data of the subject whose sputum use in this study (Table 1).

## Results

From Table 1 we assumed that male subjects were more susceptible to be suspected TB infection and that chronic cough is still the main chief complaint in TB. On the other hand, as shown in Table 2, we found that the positivity result between ZN staining only versus added 1% bleach differed significantly. From the same table we can inference that added 1% bleach have greater positivity number that the ZN only.

The Figure 1 shows that the positivity rate of bleach + ZN is better than the ZN stain only. From the same figure we can assume that 1% bleach before ZN staining have better positivity rate rather than ZN only. Based on the result in Figure 2, we can inference that the smear from bleach with ZN has clearer background, and bleach can reduce the stickiness of the sputum. The clearer background will help us to find the red bacilli more easily.

## Discussion and Conclusions

Based on the result of this study we can conclude that adding 1% bleach before preparing for ZN stain can improve the positivity rate of TB smear microscopy. From Table 2, we can see that 37 smears were positive and 29 were negative by both method (bleach and non-bleach respectively), 2 were negative by non-bleach but positive by bleach and vice versa. Both methods differ significantly, meaning that the preparation with 1% bleach was superior compare to ZN stain without bleach. The study conducted by Cattamanchi *et al*, concluded that bleach-processed smear increasing a small number of sensitivities compared to the direct smear, although there was no improvement on diagnostic accuracy. However Cattamanchi's study was limited on the heterogeneity of diagnostic accuracy for both direct and bleach microscopy.<sup>7</sup>

The higher positivity rate in 1% bleach can be seen clearly in the graph of Figure 1 (90% in 1% bleach vs 56% in non-bleach). The factor that may contribute to increase the positivity rate is the capability of bleach to digest the mucin and make the background clearer.<sup>8</sup> The study by Bonnet *et al*. in Kenya within high risk population of HIV, showed that bleach sedimentation is an effective and simple method to improve microscopy result. As we can see from Figure 2, the smear background seemed clearer than direct smear, which is consistent with study from Kenya and Nigeria, as digested smear performed well and more

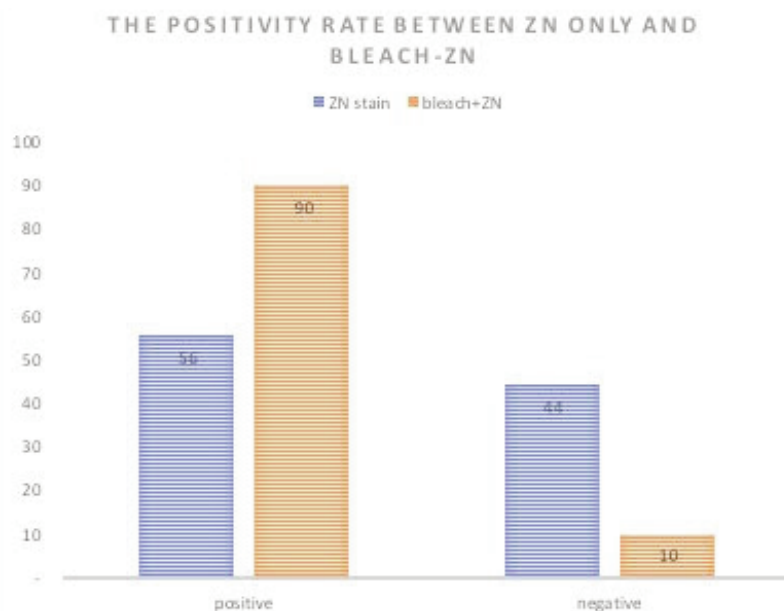
**Table 1. The subject's characteristics whose sputum were used in this study (n =70), both of them were processing with ZN only and ZN added bleach.**

NO	Characteristics	n	%	
1	Sex	Male	40	57.1
		Female	30	42.9
2	Age	< 25	4	5.7
		25-40	28	40
		> 40	38	54.3
3	Chief complain	Cough more than 2 weeks	45	64.3
		Low fever last for 2 weeks	10	14.3
		Lost of body weight	10	14.3
		Others	5	7.1

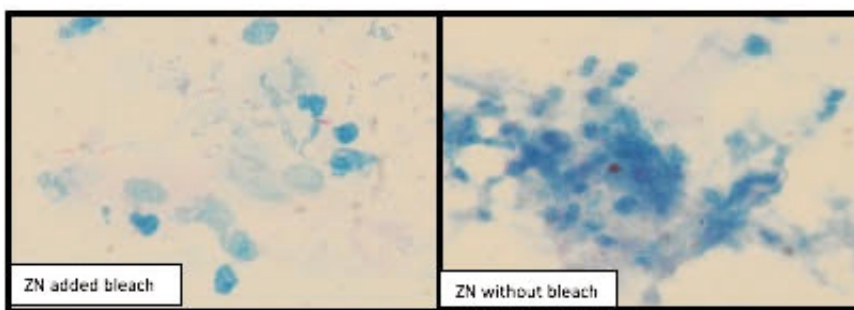
**Table 2. Cross-tabulation between ZN stain only and added 1% bleach-ZN stain.**

ZN stain	1% bleach-ZN		P*
	positive (n)	negative (n)	
Positive (n)	37	2	< 0.000
Negative (n)	2	29	

\*Significant different using Mann-Whitney U test.



**Figure 1. The positivity rate between ZN stain only and added 1% bleach-ZN stain in percentage value. (The positivity rate of ZN only is lower than bleach-ZN – 56% vs 90%).**



**Figure 2. The smear's result from added 1% bleach with ZN (left) and ZN staining only (right) from same specimen. We can see the background of 1% bleach is clearer than ZN only, so we can find the bacilli more easily.**

positive result than direct smear.<sup>8,9</sup>

David *et al.* argued that bleach-processed sputum is an alternative to current diagnostic practice in low resource settings.<sup>10</sup> The study was conducted using 5% NaOCl for 15 minutes incubation and centrifugation, thus the bleach-processed smear yield a higher result as 15.4% and there was no loss of positive result compared to non-bleach processed smear.<sup>10</sup> Compared to this study, the 1% bleach-processed group yield a 34% higher rate than the non-bleach smear, bigger number of positive result than previous study with smaller concentration and non-centrifugation method.

The method in this study is simple, inexpensive and can be completed in a short period of time, compared to other studies that use centrifugation and overnight incubation.<sup>8,10,11</sup> We only incubated the sputum for 10 minutes and no other advanced instrument was used. The main reagent, commercial bleach, is affordable and available even in very remote areas and can be used in limited setting, where tuberculosis is endemic, like Indonesia. However, as the background seems clearer because of lack of the background material, our technicians complained difficult in focusing. This limitation is similar with those mentioned in the previous study by Bonnet.<sup>8</sup> Bonnet also considered the possibility of smear washing out during slide staining, although we didn't find any difficulties regarding this issues because we only incubated for 10 minutes while Bonnet did overnight sedimentation.<sup>8</sup> The concentration in Bonnet's study was higher than our concentration, as they used 3.5% and we used only 1%, therefore this lower concentration may lead to less sputum liquefaction.

The diagnosis of tuberculosis depends on high standard biosafety for bacilli culture, however in limited resources setting, the condition is difficult to meet. We must find a safer method in conducting TB diagnostic with high positivity rate. This study doesn't need any centrifugation, so there are no aerosol sparks during laboratory work. The sample was manually shaken and the sputum container didn't open until 10 minutes after, that is our incubation period, thus the bacilli and aerosol sparks could be minimized. Bleach, is also known as an effective disinfectant, and can be used to minimize the transmission of *Mycobacterium*

*tuberculosis* bacilli to the laboratory workers.<sup>12,13</sup> Chew concluded in his study that bleach concentration before staining procedure can be used to sterilize the sputum from the *Mycobacterium tuberculosis* bacilli.<sup>5</sup> However, the limitation of this study was not counting the score of AFB, so the differentiation only based on the positive or negative appearance of the bacilli.

Other studies said that bleach-processed sputum still compatible with PCR-based method and bacilli culture in highly contaminated sputum.<sup>10,14</sup> As far as the first method is concerned, the nucleic acid component still can be examined, though the microorganism is dead,<sup>15</sup> and for bacilli culture, bleach is prominent to minimize the other bacteria instead of *Mycobacterium tuberculosis*.<sup>14,16</sup> As the bleach is inexpensive it is likely to implement the accuracy of TB smear diagnostic in developing or low income countries. By those result, we can use bleach-processed smear as an alternative for TB diagnostic in limited setting or remote area.

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