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RESEARCH

DETECTION OF *MYCOBACTERIUM TUBERCULOSIS* WITH TB ANTIGEN RAPID TEST IN PULMONARY TUBERCULOSIS PATIENTS WITH FOUR TYPES OF SPUCTUM SAMPLE PREPARATION

(Deteksi Antigen Mycobacterium Tuberculosis Menggunakan TB Antigen Uji Cepat di Pasien Tuberkulosis Paru dengan 4 Cara Preparasi Dahak)

Miftahul Ilmiah, IGAA. Putri Sri Rejeki, Betty Agustina Tambunan

ABSTRAK

Pemeriksaan mikroskopis langsung untuk mendiagnosis TB memiliki banyak keterbatasan. TB Ag Rapid Test Device merupakan metode pemeriksaan ICT TB yang mendeteksi antigen yang disekresi khas dari Regions of Difference (RD) M.tuberculosis. Penelitian sebelumnya menunjukkan uji sering tidak berjalan dengan baik. Sampel penelitian adalah 30 dahak BTA positif dengan pemeriksaan mikroskopis langsung. Dahak diberikan 4 perlakuan berbeda yaitu: perlakuan rutin sesuai tata langkah perangkat; penambahan 0,5 mL NALC 2,5% dan dahak dikocok sesuai tata langkah perangkat; perlakuan vorteks dilanjutkan pemusingan 10.000g suhu 4°C; penambahan 0,5 mL NALC 2,5%, vorteks dilanjutkan pemusingan 10.000g suhu 4°C. Hasil pemeriksaan antigen M.tuberculosis sebagai berikut: perlakuan kesatu 43,3% positif, perlakuan kedua hasil positif tinggi (96,7%), perlakuan ketiga dan keempat didapatkan hasil positif sebesar 36,7% dan 86,7%. Pemeriksaan Ag menggunakan vorteks-pemusingan dengan pemeriksaan rutin menunjukkan kesesuaian sebesar 86,6% (33,3% positif dan 53,5% negatif) dengan nilai Kappa 0,724 (p<0,0001). Perlakuan 2 (penambahan 0,5 mL NALC 2,5%) dengan perlakuan 4 (penambahan NALC 2,5%-vorteks-pemusingan) menunjukkan kesesuaian sebesar 90% (86,7% positif dan 3,3% negatif) dengan nilai Kappa 0,366 (p=0,010). Pemberian pretreatment 0,5 mL NALC 2,5% dapat digunakan untuk meningkatkan hasil positif kit TB Ag Rapid Test Device untuk mendiagnosis tuberkulosis paru.

Kata kunci: *TB Ag rapid test device, M.tuberculosis, sputum, regions of difference*

ABSTRACT

Direct microscopic examination to diagnose TB has many limitations. TB Ag Rapid Test Device is an ICT examination method detecting TB-specific antigen secreted from the regions of difference (RD) of *M.tuberculosis*. Previous research indicated that tests can not be always performed well. Samples were 30 positive sputum smears by direct microscopy sputum. Sputum samples underwent 4 treatments: routine treatment according to the kit procedure; addition of 0.5 mL 2.5% NALC and whipped according to the kit procedure; vortex treatment followed by centrifugation 10,000g temperature 4°C; addition of 0.5 mL 2.5% NALC, followed by vortex-centrifugation 10,000g temperature 4°C. The results of the antigen examination using TB Ag Rapid Test Device were as follows first routine treatment is 43.3% positive, second treatment had a high positive result (96.7%), third and fourth treatment had positive result 36.7% and 86.7%. Examination of Ag using vortex-centrifugation with a routine examination showed a compatibility of 86.6% (33.3% positive and 53.5% negative) with a Kappa value of 0.724 (p<0.0001). Treatment 2 (addition of 0.5 mL 2.5% NALC) with treatment 4 (addition of 0.5 mL 2.5% NALC- vortex-centrifugation) demonstrated a compatibility of 90% (86.7% positive and 3.3% negative) with a Kappa value of 0.366 (p=0.010). The addition of 0.5 mL 2.5% NALC pretreatment can be used to increase the positive outcome of TB Ag Rapid Test Device for diagnosing pulmonary tuberculosis.

Key words: TB Ag rapid test device, *M.tuberculosis*, sputum, regions of difference

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INTRODUCTION

Tuberculosis (TB) is a health problem for millions of people every year and considered as the second cause of death.[1] Indonesia has established the alleged diagnosis of tuberculosis by staining acid-resistant rod. To make the result positive, the examination should use a microscope requiring approximately 5,000 bacteria/mL of sputum. However, this method depends on the workload, skills, and motivation of technicians in reading sample preparation.[2,3]

Making culture in Lowenstein Jensen (LJ) media as the gold standard examination on the bacillus of *Mycobacterium tuberculosis,* moreover, will take long time.[4] Polymerase Chain Reaction (PCR) or nucleic probes, on the other hand, is more impractical and expensive, as well as requires skilled staff and special laboratory facilities.[4,5] Meanwhile, immunochromatographic test (ICT) is an examination technique that is faster and more inexpensive, as well as does not require special equipment and expertise.[4,6]

TB Ag Rapid Test Device is a simple method of ICT used in TB examination with sputum samples. This method detects specific secreted antigens (CFP-10, ESAT-6, MPb64 and prophage phiRv1) derived from Regions of Difference (RD) of *M.tuberculosis* (RD1, RD2, RD3).[7,8]

Kartika in 2011 proved that TB Antigen Rapid Test Device is an antigen examination, analyzing more quickly. The microscopic sensitivity of Acid-resistant rod (83.8%) was higher than the one of TB Antigen Rapid Test Device (72.6%) due to the different viscosity of sputum among patients. Dilution of greater sputum samples is actually required on very thick sputum, but determining the precise number of the samples in order to make the test run well is very difficult. A research suggests TB Antigen Rapid Test Device by administering a pretreatment in sputum that do not destroy epitope of the antigens.[8]

Liu in 1999, furthermore, separated mucous in the sputum of patients with asthma, and then obtained an increase in eosinophil count. Next, the sputum was mixed using vortex, added with 0.1% dithiothreitol before centrifuged at 10,000 g at a temperature of 4°C and incubated for 15 minutes.[9] Some researchers, on the other hand, added mucolytic, such as 2.5% N-acetyl L-cysteine (NALC) or 0.1% dithiothreitol to break down mucous in tuberculoma of *M.tuberculosis*.[10,11]

Therefore, this research will focus on the compatibility of TB Ag Rapid Test Device for diagnosing pulmonary tuberculosis with routine manual shaking technique according to the procedures of reagent kit, compared with: routine manual shaking technique with the addition of 0.5 mL of 2.5% NALC before the examination; shaking technique with a vortex-centrifugation at a speed of 10,000g at a temperature of 4°C before the examination; shaking technique with the addition of 0.5 mL of 2.5% NALC and a vortex-centrifugation at a speed of 10.000g before the examination.

METHODS

This research was an observational research with cross sectional design. Specimens used in this research were microscopically positive sputum of acid-resistant rod derived from 30 TB patients in Surabaya Lung Hospital from October 2013 to March 2014. Next, examination of TB Ag Rapid Test Device was performed at the research and development unit (R&D) of Department/Installation of Clinical Pathology in Faculty of Medicine of Airlangga University/ Dr. Soetomo Hospital. ICT method was used in this research with TB Ag Rapid Test Device (JD Biotech, Taiwan) and the lot number of TB-131 013 with ED October 12, 2015, read by two analysts and one doctor. The results then were analyzed statistically using Kappa test.

Next, sputum samples were treated differently. First, each of four tubes were given with 1 mL of buffer solution and 0.2 to 0.3 mL of sputum. In Tube 1, the sample then was mixed for 30–60 seconds and waited (for 30 minutes). In Tube 2, the sample was added with 0.5 mL of 2.5% N-acetyl L-cysteine. After abandoned for 15 minutes, the sample was shaken manually for 0.5–1 minutes and waited for 15 minutes. In Tube 3, the sample was mixed using vortex (for 10 minutes), waited for 15 minutes and then centrifuged (for 10 min) at a speed of 10,000 g at a temperature of 4°C. In Tube 4, the sample was added with 0.5 mL of 2.5% N-acetyl L-cysteine, waited for 15 minutes, mixed with vortex for 10 minutes and then centrifuged for 10 minutes at a speed of 10,000g at a temperature of 4°C. Next, supernatant of each sample were taken about 2–4 drops or 100–200 mL and put in the area of S in the kit. The results then were read after 15 minutes.

RESULTS AND DISCUSSION

The number of TB patients with positive acid-resistant rod was 30 people, consisted of 50% males and 50% females with a mean age of 39,067 (mean±SD = 39,067+11,980). Characteristics of the

research subjects involving age, gender and percentage of acid-resistant rod samples microscopically can be seen in Table 1.

Table 1. Characteristics of the research subjects

Variables	Frequency	Percentage
Age (years)		
≤20	3	10.0
21–30	6	20.0
31–40	5	16.7
41–50	11	36.7
51–60	5	16.7
Sex		
Males	15	50.0
Females	15	50.0
BTA		
1+	15	50.0
2+	7	23.3
3+	8	26.7

The results of the examination on Ag of *M.tuberculosis* using TB Ag Rapid Test Device can be seen in Table 2. The highest positive result was found in Tube 2 with the addition of 2.5% NALC as shown in Table 2.

The compatibility of the examination results on Ag of *M. tuberculosis* using the addition of 2.5% NACL (Tube 2) compared to routine procedures (Tube 1) was 46.6% (43.3% for the positive one and 3.3% for the negative one). The value obtained from Kappa test was 0.051 (p=0.374). It means that the treatment using the addition of 2.5% NALC was not compatible with the treatment using routine procedures as shown in Table 3.

The compatibility result of the examination on Ag of *M.tuberculosis* using vortex-centrifugation in 30 TB patients with positive acid-resistant rod (Tube 3) compared to routine procedures (Tube 1) was 86.6% (33.3% for the positive one and 53.3% for the negative one). The value obtained from Kappa test was 0.724 (p <0.0001). It indicates that Tube 1 using routine procedure was compatible with Tube 3 using vortex-centrifugation, as shown in Table 4.

The compatibility of the examination results on Ag of *M.tuberculosis* using the addition of 2.5% NACL (Tube 2) compared to vortex-centrifugation (Tube 3) in those 30 TB patients with positive acid-resistant rod showed 40.0% (36.7% for the positive one and 13.3% for the negative one). The value obtained from Kappa test was 0.039 (p=0.439). It means that the treatment using the addition of 2.5% NALC (Tube 2) was not compatible with the treatment using vortex-centrifugation (Tube 3) as seen in Table 6.

Table 2. The results of the examination on antigen of *M.tuberculosis* using TB Ag Rapid Test Device

Treatment	Examination	Positive	Negative
1	Routine	13 (43.3)	17 (56.7)
2	2.5% NALC	29 (96.7)	1 (3.3)
3	Vortex–centrifugation	11 (36.7)	19 (63.3)
4	2.5% NALC + vortex-centrifugation	26 (86.7)	4 (13.3)

Table 3. The compatibility results of the examination on antigen of *M.tuberculosis* between 2.5% NALC treatment and routine procedure treatment

2.5% NALC	Routine examination			Kappa test	
	Positive	Negative	Total	Kappa	P value
Positive	13 (43.3)	16 (53.3)	29 (96.7)	0,051	0.374
Negative	0 (0.0)	1 (3.3)	1 (3.3)		
Total	13 (43.3)	17 (56.7)	30 (100.0)		

Table 5. The compatibility results of the examination on antigen of *M.tuberculosis* between 2.5% NALC + vortex-centrifugation treatment and routine procedure treatment

2.5% NALC-vortex–centrifugation	Routine examination			Kappa test	
	Positive	Negative	Total	Kappa	P value
Positive	12 (40.0)	14 (46.7)	26 (86.7)	0.089	0.427
Negative	1 (3.3)	3 (10.0)	4 (13.3)		
Total	13 (43.3)	17 (56.7)	30 (100.0)		

Table 4. The compatibility results of the examination on antigen of *M.tuberculosis* between vortex-centrifugation treatment and routine procedure treatment

Vortex–centrifugation	Routine examination			Kappa test	
	Positive	Negative	Total	Kappa	P value
Positive	10 (33.3)	1 (3.3)	11(36.7)	0.724	<0.0001
Negative	3 (10.0)	16(53.3)	19 (63.3)		
Total	13 (43.3)	17 (56.7)	30 (100.0)		

Table 6. The compatibility results of the examination on antigen of *M.tuberculosis* between 2.5% NALC treatment and vortex–centrifugation treatment

2.5% NALC	Vortex–centrifugation			Kappa test	
	Positive	Negative	Total	Kappa	P value
Positive	11 (36.7)	18 (60.0)	29 (96.7)	0.039	0.439
Negative	0 (0.0)	1 (3.3)	1 (3.3)		
Total	11 (36.7)	19 (63.3)	30 (100.0)		

Table 7. The compatibility results of the examination on antigen of *M.tuberculosis* between 2.5% NALC + vortex–centrifugation treatment and vortex – centrifugation treatment

2.5% NALC-vortex–centrifugation	Vortex–centrifugation			Kappa test	
	Positive	Negative	Total	Kappa	P value
Positive	11 (36.7)	15 (50.0)	26 (86.7)	0.164	0.102
Negative	0 (0.0)	4 (13.3)	4 (13.3)		
Total	11 (36.7)	19 (63.3)	30 (100.0)		

The compatibility of the examination results on Ag of *M.tuberculosis* using the addition of 2.5% NACL (Tube 2) compared to vortex-centrifugation (Tube 3) in those 30 TB patients with positive acid-resistant rod showed 50.0% (36.7% for the positive one and 13.3% for the negative one). The value obtained from Kappa test was 0.164 (p=0.102). It means that the treatment using the addition of 2.5% NALC (Tube 2) was not compatible with the treatment using vortex-centrifugation (Tube 3).

Moreover, the compatibility of the examination results on Ag of *M.tuberculosis* using the addition of 2.5% NACL (Tube 2) compared to 2.5% NALC + vortex -centrifugation (Tube 4) in those 30 TB patients with positive acid-resistant rod showed 90% (86.7% for the positive one and 3.3% for the negative one). The value obtained from Kappa test was 0.366 (p=0.010). It indicates the treatment using the addition of 2.5% NALC (Tube 2) was not compatible with the treatment using 2.5% NALC + vortex –centrifugation (Tube 4).

Table 8. The compatibility results of the examination on antigen of *M.tuberculosis* between 2.5% NALC treatment and vortex–centrifugation treatment

2.5% NALC	NALC 2,5% + vortex–centrifugation			Kappa test	
	Positive	Negative	Total	Kappa	P value
Positive	26 (86.7)	3 (10.0)	29 (96.7)	0.366	0.010
Negative	0 (0.0)	1 (3.3)	1 (3.3)		
Total	26 (86.7)	4 (13.3)	30 (100.0)		

Research on TB Ag Rapid Test Device actually has been done by some researchers to analyze its sensitivity and specificity. Kartika in 2011, for instance, declared the sensitivity and specificity of TB Ag Rapid Test Device were respectively 72.6% and 90.9%.[8] Unlike the previous research, a research conducted by Prasetyo in 2013 states that the sensitivity and specificity of TB Ag Rapid Test Device were not good, about 43.7% and 76.2%.[8,12]

Of the 30 TB patients with positive acid-resistant rod in this research, the ratio between male and females was the same, namely 50%: 50%. The minimal age of those patients was 19 years, while the maximal one was 59 years with a mean age of 39.067 (mean±SD = 39.067+11.980). In other words, most patients with TB in this research was at the productive age.[13]

Reading of microscopic preparations, furthermore, is actually related to quality of sputum and skills of analysts. Besides, workload of analysts can also affect low quality of diagnostic services. The International Union Against Tuberculosis and Lung Disease (IUATLD), consequently, recommends an average of 20 stocks per technician per working day.[8,13]

The results of Kappa test on the three readers in this research showed very high compatibility results among the three readers. One of the microscopic results of sputum samples obtained was +2, but those three readers interpreted this reading result of TB Ag Rapid Test Device of all the treatments as negative one. It may be caused by Mycobacterium Other Than Tuberculosis (MOTT)/nontuberculosis mycobacteria and Hook Effect phenomenon that is antigen excess saturating antibody sandwich so that the configuration cannot be formed and lead to negative results.

TB Ag Rapid Test Device is an ICT test which detects specific proteins of *M.tuberculosis* encoded, namely RD1, RD2, RD3. Some RD antigens are encoded by the genome of *M.tuberculosis, M.africanum* and *M.bovis*, but not obtained at all substrains of BCG and other environmental mycobacteria.[14] Three genomic RDs (Regions of Difference) are RD1, RD2 and RD3 removed at BCG. Distribution of RD on MTC members is RD1 in *M.tuberculosis, M.africanum, M.bovis* and *M.microti*, but none at BCG of *M.bovis*, RD2 in *M.tuberculosis, M.africanum, M.bovis* and *M.microti* and partly at BCG of *M.bovis*, as well as RD3 only in *M.tuberculosis* and *M.bovis*, but none at BCG of *M. bovis*.[15,16]

Principles of Ag TB Rapid Test Device examination involve double antibodies chromatographic lateral flow immunoassay. A specific antibody against analyte tracked is immobilized on a nitrocellulose membrane and then attached to a conjugate antibody labeled. Therefore, samples containing specific Ag of *M.tuberculosis* will form both antigen-antibody complexes in the immobilization zone and color according to the level of the analyte (antigens) in the samples. Hook Effect phenomenon then occurs when the levels of the analyte in the samples are excessive (excessive antigens) exceeding the levels of antibody binding (capture Ab) contained in capture line, causing several microspheres not tied (passes) in the capture line and flowing continually to a second line of immobilized antibody, namely control line.[17] In this research, the possibility of excessive levels of analyte led to negative results.

Error rate of acid-resistant rod preparation microscopically in a sampling of 2011 was 2% in the first quarter, and 0% in quarters 2, 3 and 4. Meanwhile, in 2012, it was 0.9% in the second quarter. One of the reading results of the microscopic acid-resistant rod samples obtained was +2, but the test using TB Ag Rapid Test Device kit had a negative result on all treatments. However, the researchers did not regard this as a microscopic reading error. Error rate of the smear preparation in sampling sites was less than 5% in the previous year. This research used sputum samples of positive acid-resistant rod without using any other supporting data (photograph piston, culture LJ). The diagnosis of pulmonary tuberculosis in adults in Indonesia is confirmed by the discovery of acid-resistant rod in sputum smear on microscopic examination. Test results can be confirmed as positive one if at least two of three specimens of SPS have positive acid-resistant rod.[13]

The compatibility of the examination results on Ag of *M.tuberculosis* using vortex-centrifugation

(Tube 3) compared to a routine examination (Tube 1) was 86.6%. The Kappa value obtained from Kappa test was 0.724 ($p<0.0001$). It means that Tube 1 was compatible with Tube 3. Meanwhile, the compatibility of the examination results using treatment 1 and treatment 2 was low positive, namely 43.3% and 36.7%. Examination of Ag Rapid Test Device without pretreatment actually depends on homogenization techniques. The previous researchers added buffer and shook strongly to get better results. Besides, the addition of larger buffer is needed to reduce the viscosity of the very thick sputum. The secretion of organisms from mucin or cells can also be enhanced by using vortex. This process will cause damage to the cells of *M.tuberculosis,* so many proteins will be secreted.[8,18]

In addition, the reading results of TB Ag Rapid Test Device using routine procedures (Tube 1) were 43.3% for the positive one and 56.7% for the negative one. The low results were possibly caused by Ag of *M.tuberculosis* still trapped in the viscous mucous. Sputum is the result of tracheobronchial secretions. Tracheobronchial secretions are derived from mucous glands and goblet cells. Goblet cells produce thick mucin. Besides, the secretions also contain mast cells, eosinophils, and plasma cells. The physical properties of sputum is viscoelastic. Sputum consistency depends on the molecular structure of glycoproteins and water level. Meanwhile, the chemical composition of sputum consists of 95% water and 5% solids. The solid form is primarily carbohydrates, proteins, lipids and DNA.[8]

Moreover, the reading results of TB Ag Rapid Test Device using vortex-centrifugation (Tube 3) showed positive one, at least 36.7%. Vortex treatment was expected to replace manual shaking (Tube 1) and then centrifugation was conducted to separate the mucous with TB antigens. Thus, the low result may be caused by antigens possibly trapped in mucous, so they would be sedimented after centrifugation was done.

Furthermore, the reading results of TB Ag Rapid Test Device using the addition of 0.5 mL of 2.5% NALC (Tube 2) showed positive one with the highest percentage of 96.7%. Previous research states that the addition of pretreatment on samples that did not destroy the epitope of TB antigens is very important.[8,18] N-acetyl L-cysteine (NALC) containing thiol in the sulfhydryl group playing a direct role as a reactive oxygen species scavenger, so releasing the disulfide bond and breaking mucous.[19]

The addition of 0.5 mL of 2.5% NALC (Tube 2) in the first 15 minutes of incubation would release the disulfide bonds and break the mucous, so antigens of *M.tuberculosis* would be released from the mucous. Next, manual shaking will homogenize and increase antigens released. The second incubation then was conducted for 15 minutes. The aim of this second incubation is to make mucous and antigens that will be examined sedimented and antigens released higher.

In addition, the compatibility result of the examination on Ag of *M.tuberculosis* in Tube 2 compared to Tube 4 was 90% (86.7% positive and 3.3% negative). Both of these treatments also generated a high positive value after the addition of 2.5% NALC. It indicates that the addition of 2.5% NALC can be able to replace either routine examination or vortex-centrifugation. The addition of 0.5 mL of 2.5% NALC (Tube 2) compared to the addition of 0.5 mL of 2.5% NALC + vortex-centrifugation (Tube 4) showed the Kappa value of 0.366 (less score). It means that the two treatments was not compatible and interchangeable since the compatibility results of the two treatments had to be 0.010.

The treatment using 2.5% NALC + vortex-centrifugation (Tube 4), moreover, generated lower antigens than Tube 2. Several possibilities are suspected as the cause, namely: 1) the longer incubation period with 2.5% NALC in Tube 2 (10 minutes and 15 minutes after the shaking) than Tube 4 (only 10 minutes) in which after the vortex-centrifugation treatment, the examination of Ag kit TB Rapid Test Device was directly performed, so chance for releasing antigen was reduced; 2) the short time of the centrifugation (only 10 minutes) making the separation less perfect and protein in the mucous sedimented.

Similarly, Ratnam in 1986 showed that sputum centrifuged faster and longer can improve the mean number of Mycobacteria growth in LJ media. The centrifugation duration of 10 minutes, 15 minutes and 20 minutes at the centrifugation speed of 2,007 g, 3,005 g and 3,895 g generated the highest number of Mycobacteria growth in the duration of 20 minutes at the speed of 3,895 g, therefore, the highest number of Mycobacteria growth obtained in sediments was 80.2%.[20]

Actually, there are so many researches using a centrifugation speed of 10,000g at a temperature of 4°C. For instance, Liu in 1999 separated mucous in the sputum of patients with asthma. By using vortex, the sputum then was added with 0.1% dithiothreitol before centrifuged at a centrifugation speed of 10,000 g at a temperature of 4°C, and then incubated for 15 minutes.[9] Beck in 2005 also examined antigens of *M.tuberculosis* (ESAT-6, MPB 64, A60 and Mtb 81) using a centrifugation speed of 10,000 g for 30 minutes at a temperature of 4°C.[9,21] These researchers speculate if the centrifugation was conducted at the speed of

10,000 g at a temperature of 4°C, but with the longer time before incubation process, the number of antigens would be higher.

CONCLUSION AND SUGGESTION

Based on the results of this research, it can be concluded that the samples that did not get additional pretreatment (0.5 mL of 2.5% NALC) showed poor results. It means that adequate shaking is very important in processing samples. The addition of 0.5 mL of 2.5% NALC continued with manual shaking can give the best results in determining antigens of *M.tuberculosis* in sputum and also can replace routine examination in accordance with the procedures of existing kit. Further research needs to be done using a larger sample. In addition, research on the effects of the relative centrifugal and centrifugation time with the addition of pretreatment needs to be done.

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