

Validity of Mycobacterium Tuberculosis Antigens Cocktail (ESAT-6, CFP-10, MPT 64) Serum Test in Diagnosing Adult and Pediatric Tuberculosis

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Abstract: *Background:* The diagnosis of tuberculosis relies on the isolation of *Mycobacterium tuberculosis* (*M. tuberculosis*) from sputum culture but in pediatric patient it is difficult to take the sputum. For individuals such as children who are unable to produce sputum, invasive procedures may become necessary when noninvasive methods do not permit a diagnosis. The aim of this study was to determine the validity of *M. tuberculosis* antigens cocktail ESAT-6, CFP-10 and MPT64 (TB Antigens Cocktail) from serum specimens in Adult TB with Ogawa culture as a gold standard, and from serum specimen of pediatric TB patients with TB Scoring system as a gold standard. *Subject and method:* This is a cross sectional descriptive observational study. This study was held in Dr Hasan Sadikin General Hospital Bandung, Indonesia. Subjects were divided into two groups: adult TB and Pediatric TB. The sputum specimens from the first group were obtained Ziehl-Neelsen (ZN) stain and *M. tuberculosis* culture (Ogawa). The TB Antigens Cocktail rapid Immunochromatography (ICT) was performed on all serum samples from both groups. *Result:* A total of 68 Adult TB were enrolled in this study, the sensitivity, specificity, PPV and NPV of TB Antigens Cocktail were 24.2 %, 85.7%, 61.5% and 54.5%, respectively. From 61 children suspected with TB disease, the sensitivity, specificity, PPV and NPV of TB Antigens Cocktail were 30.7%, 86.3%, 80.0% and 41.3% respectively. *Conclusions:* This study shown, Immunochromatography TB Antigens Cocktail (ESAT-6, CFP-10 and MPT64) from serum sample has good specificity for diagnosing tuberculosis.

Keywords: Adult pulmonary tuberculosis, Pediatric tuberculosis, TB antigen cocktail, serum.

1. Introduction

Tuberculosis (TB) remains a major global health problem, in 2015 was one of the top 10 cause of death worldwide.(1) The early diagnosis of pulmonary tuberculosis (PTB) is very important in reducing morbidity and mortality. The diagnosis of PTB is based on sputum microscopy identification for acid-fast bacillus (AFB) and culture of *M. tuberculosis*. However there are 30% of patients who can not produce sputum sample as in adult PTB with HIV and pediatric tuberculosis patient.(2-4) Approximately 1 million children develop TB disease each year and at least 14% die in where the majority of these children are never diagnosed or treated for their TB disease.(5) Without adequate treatment, children with TB, especially those younger than 5 years, are at high risk of death.(6) Diagnosis of pediatric TB needs to be more in the clinician's effort in obtaining medical history and physical examination because of the paucibaciller population of *M. tuberculosis* and the unspecific symptoms of disease. For individuals such as children who are unable to produce sputum (natural or induced), invasive procedures may become necessary when noninvasive methods do not permit a diagnosis. In the absence of a positive culture, the strongest evidence for TB in a child is recent exposure to an adult with active disease. The tuberculin skin test and chest radiography may be used to provide supportive information. Therefore Scoring

system has been recommended by several government institution as helping tool to diagnose it.(7, 8)

Diagnosis of TB using microscopy examination is less sensitive and can be found Mycobacterium other than tuberculosis. While, the isolation and identification of *M. tuberculosis* using culture is time consuming and require high skilled experts and safety precautions.(9, 10) The polymerase chain reaction (PCR) method is very expensive to apply in poor resource settings.(11, 12) Recently there is a test provided results by detecting the *M. tuberculosis* antigens cocktail ESAT-6, CFP-10, and MPT-64 using the lateral flow principle. Previous studies were found the *M. tuberculosis* antigens cocktail ESAT-6, CFP-10, and MPT-64 in several body fluid.(13-16) *Mycobacterium tuberculosis* secretion of ESAT-6, CFP-10 and MPT-64 antigens coded by Regions of differences 1 (RD1) and RD2 genes. These regions of differences are located in the genome of *M. tuberculosis* but somehow are absent in many of the environmental mycobacteria.(17) Early secretory antigenic target-6 (ESAT-6) is expressed early during *M. tuberculosis* infection and it is essential for the bacteria to survive and spread in vivo.(18) In other hand, culture filtrate protein-10 (CFP-10) has been identified to be earliest protein produced by *M. tuberculosis* during culture in bacteriological media.(19) *M. tuberculosis* protein 64(MPT-64) is a secreted protein of *M. tuberculosis* and elicits specific response against *M. tuberculosis*.(20) Both

ESAT-6 and CFP-10 are proofed to down regulate Reactive Oxidative Species (ROS) production inside macrophages. This condition has lead to inhibited Nuclear Factor κ B (NF- κ B) activation and transcriptional property.(21) Numbers of studies suggest that ESAT6 protein induces apoptosis in human monocytic cell line, they also suggested that ESAT6 could be inducing pore-formation on the macrophage and dendritic cell membranes resulting the spread of *M. tuberculosis* antigen and found in the serum.(9, 13, 21-23)

Based on that situation, the aim of this study was to determine the validity of *M. tuberculosis* antigen cocktail ESAT-6, CFP-10 and MPT64 from serum specimens in adult PTB and pediatric TB patients.

2. Material and Methods

This study involved two groups of tuberculosis patients. The first group were adult patients with suspected PTB according to the International Standard of Tuberculosis (ISTC) who presented at the outpatient TB clinic of Dr. Hasan Sadikin General Hospital, Bandung, Indonesia. The second group was new patients aged ≤ 14 year who came to Children's Clinic with diagnose based on TB Scoring system, score ≥ 6 was defined as pediatric TB. The study excluded participants who under Oral Anti Tuberculosis treatments, those with malnutrition or malignancy, which would act as confounding factors in the performance of the test. Patients or their parents have signed written informed consent for data to be used in this analysis and The Ethical Committee of the Faculty of Medicine, Universitas Padjadjaran-Dr Hasan Sadikin General Hospital, Bandung Indonesia, has approved the process.

This study was a cross sectional descriptive observational study. The sputum specimens from the first group were obtained for direct examination of acid-fast bacilli (AFB) by Ziehl-Neelsen (ZN) (ST Reagensia Company, Jakarta, Indonesia) staining using 100 x oil immersion microscopy. The remaining sputum specimens were decontaminated by a standard N-acetyl-Lcystein (NaLC)-NaOH method and concentrated by centrifugation at 3000 x g for 15 minutes where for each patient producing one sputum culture. Sputum specimens from patients who had provided ≥ 1 specimen were mixed before decontamination. The resulting sediments were then resuspended and inoculated into Ogawa medium. The growth of the bacteria was read every week until 8 week. According to the Standard operating procedure. The niasin test was performed on all positive cultures to identify *M. tuberculosis*. All group were following a vena puncture, 3 mL of blood is obtained by flebotomy in the cubital vein through disposable syringe. The blood is inserted into the vacutainer tube, allowing for it to stand for 30 minutes in a perpendicular position until the freezing point is subsequently centrifuged at a rate of 3000 g for 10 minutes. The formed serum later be separated and inserted into a microtube then heating at 56°C for 30 minutes inside the heating block before examination. We excluded the contamination culture and hemolysis or jaundice serum. The TB Antigens Cocktail

Immunochromatography (ICT) was performed on all samples by following the manufacturer's instructions Jei Daniel Biotech Corp., Taiwan. This tool has been used in previous research with good results.(24) The solution pipetted to 100ul of heating serum specimen and 100uL of sample buffer was added to the tube and mixed well for 30 minutes. Four drops (200 uL) were applied into "S" region of card for testing and the result is read after 15 minutes. The result was considered positive if in addition to a pink colored control band, a distinct pink colored band also appear in test region. It was considered negative only if monochrome band appears on the control region. The test result was considered invalid when there was a total absence of color in both regions, indicating procedure errors and/or test reagent deterioration. The study was conducted at the Clinical Pathology Laboratory of Dr. Hasan Sadikin General Hospital.

Performance of serum TB Antigens Cocktail test to diagnose Adult TB was determine using sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) by comparing result with the gold standard test for active TB, the sputum Ogawa culture. Since the sputum is difficult to obtain from children then the performance of serum TB Antigens Cocktail test to diagnose pediatric TB was determine using sensitivity, specificity, PPV, NPV by comparing result with the TB Scoring system as a gold standard. The positivity rate of TB antigens cocktail immunochromatography (ICT), AFB microscopy, mycobacterial culture and Tuberculin skin test (TB Scoring system) was expressed in percentage.

3. Results

From July 2014 to October 2014, there were 68 suspects adult PTB. Sputum culture was positive in 33 (48%) samples, 35 (52%) samples were negative (Figure 1). Of the culture positive, 32/33 (96,9%) were sputum AFB positive. Five participants who were negative by the sputum culture were positive by TB Antigens Cocktail serum. Of these all participants were AFB positive. (Figure 1).

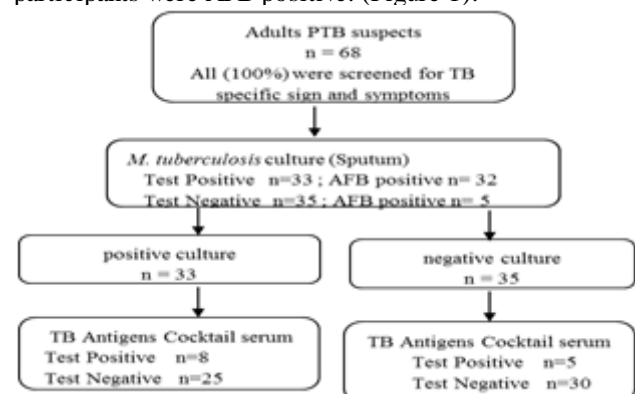


Figure 1: Tuberculosis diagnostic flow and diagnostic results of Adults PTB suspects

From January 2015 to February 2016, there were 61 suspects pediatric TB subjects. Among them, 44 had current cough (72,1%), 42 were lymph nodes positive (68,9%), 41

suspect had an unexplained fever ≥ 2 weeks (67%), 36 has a family history with positive AFB (59%), 25 had a Tuberculin skin test positive (41%), one suspect suffer joint or bone swelling (2%), 42 had a Nutrition $<60\%$ (68,9%) and Radiography suggestive with TB were 39 (64%). According to TB Scoring system, score ≥ 6 was defined as pediatric TB. There are 39 (63,9%) subjects with TB Scoring system ≥ 6 and 22 (36.1%) with TB Scoring system <6 (Figure 2).

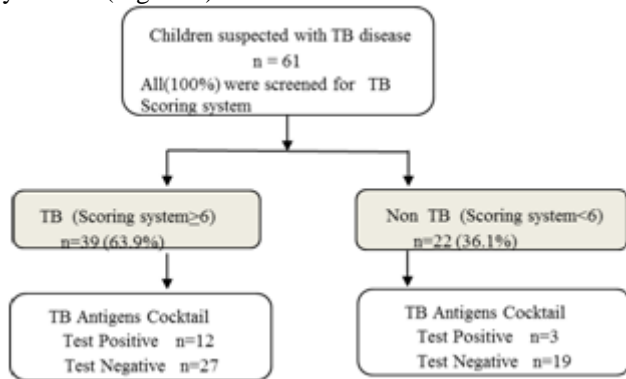


Figure 2: Tuberculosis diagnostic flow and diagnostic results of Pediatric TB suspects

The TB Antigens Cocktail test is positive in 28 (21.7%) out of the 129 study participants.

Table 1: Positivity Rate of TB Diagnostic Tests on Serum Specimens

Diagnostic test	Positivity Rate			
	Adults TB		Pediatic TB	
	Number	%	Number	%
TB antigen cocktail	13/68	19.2	5/61	24.5
AFB microscopy	37/68	54	-	-
sputum culture	33/68	48	-	-
Tuberculin skin test	-	-	25/61	40.9

The positivity rate of TB Antigens Cocktail test in adult and pediatric TB were 19.2%, 24.5%, respectively. While positivity rate of AFB microscopy, *M. tuberculosis* sputum culture and Tuberculin skin test were 54%, 48% and 40%, respectively. (Table 1).

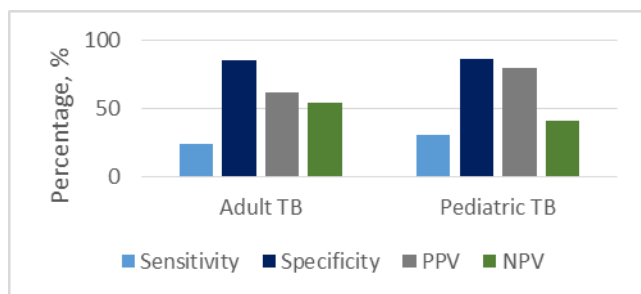


Figure 3: Validity of TB Antigens Cocktail from Serum Specimens

The sensitivity, specificity, PPV and NPV of Determine TB Antigens Cocktail serum specimens test from adult TB patients group by *M. tuberculosis* Culture to be 24.2 %, 85.7%, 61.5% and 54.5%, respectively. The sensitivity, specificity, PPV and NPV of Determine TB Antigens Cocktail serum specimens test from Pediatric TB patients

group by TB Scoring system were 30.7%, 86.3%, 80.0% and 41.3% respectively (Figure 3).

4. Discussion

This study used TB antigen Cocktail including: ESAT-6, CFP-10 and MPT64 because a combination of these proteins improve the sensitivity and specificity.(14) *Mycobacterium tuberculosis* secretion of ESAT-6, CFP-10 and MPT-64 antigens which is coded by RD1 and RD2 genes. The RD1 locus presents in clinically pathogenic strains of *M. tuberculosis* and *M. bovis*, but is deleted in *M. bovis* BCG (bacillus Calmette-Guerin) vaccine strains.(25) This is very important because all pediatric TB subjects had a history of BCG immunization in align with the national immunization program established by the Indonesia government. Therefore, the vaccination status is not an interfering factor for the research.

The positivity rate of TB Antigens Cocktail test from pediatric TB subjects was lower than Tuberculin skin test (24,5% vs 40,9%). Tuberculin Skin test is a tool for TB screening which has a low specificity and frequent reactions in low-risk individuals.(26) A false positive result may be caused by nontuberculous mycobacteria or previous administration of BCG vaccine.(27) Tuberculin Skin test do not distinguish between active TB and latent *M. tuberculosis* infection, so their diagnostic utility for active TB would be limited in population with high prevalence of latent infection.(21) Another disadvantages of tuberculin skin test is must wait for several days to be able to see the result so follow-up visit required.(28) Determine TB Antigens Cocktail test detected TB in 3 (13.6 %) individuals who's TB was missed by Scoring system. This suggests that determine TB Antigens Cocktail may be used to the challenges in diagnosing TB using Scoring system (Figure 2).

In adult TB, the positivity rate of AFB (54%) and *M. tuberculosis* sputum culture (48%) were higher than TB antigen cocktail (19.2%). This shows that in adult TB patients who can produce sputum, AFB microscopy better than serum examination. However TB Antigens Cocktail test from serum sample may be used for adult TB patients who can not produce sputum because it can detect 19,2 % of diseases. Five participants who were positive by TB Antigens Cocktail serum and AFB, were negative by the sputum culture. This can be due to mycobacterial culture was imperfect gold standard, losses in viability of tubercle bacilli will gave a negative result.

We found the sensitivity and specificity of TB Antigens Cocktail test were 0%, 100% respectively. This low sensitivity might be due to the TB antigens cocktail forming antigen-antibody complexes, that can not be bound by monoclonal antibodies present in the rapid ICT strip. ESAT-6, CFP-10 and MPT-64 antigens secreted into the macrophages during early stages of infection, these imunodominat antigens could be inducing pore-formation on the macrophage and dendritic cell membranes resulting

the spread of *M. tuberculosis* and antigen which it could enter the blood-stream.(29) These antigens released into the circulation where they bind to specific antibodies to form large immune complexes.(30) Another possibility is a free TB antigens cocktail released from *M. tuberculosis* organisms into the circulation are not antibody bound, but diluted in serum so that it is below the test's detection limit. Previous studies have found that by heating serum at 56°C for 30 minutes can eliminate the activity of antibodies, based on these studies we heated all serum samples before the ICT test.(31, 32) The modified serum could increase the sensitivity 24,2% and specificity 85,7% in adult TB patients and in Pediatric TB patients were 30.7%, 86.3%, respectively. This test had low sensitivity but high specificity in both groups. We found lower sensitivity and specificity than in a study conducted by Kalra et al. which were 95% and 95%, respectively. This difference might be due to the fact that they used enzyme linked immunoassay (ELISA) to detect serum TB Antigens.(14)

5. Conclusion

This study shown, the TB Antigens Cocktail (ESAT-6, CFP-10 and MPT64) test using serum sample has good specificity for diagnosing tuberculosis but a low sensitivity. Therefore the positive result of TB Antigens Cocktail using serum as additional test for scoring system could help in diagnosing TB in children, however the negative result of TB Antigens Cocktail test could not rule out the diagnosis of TB and require further examination.

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