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Published in:

Open Access Macedonian Journal of Medical Sciences

Publication status and date:

Published: 21/08/2021

DOI (link to publisher):

[10.3889/oamjms.2021.6558](https://doi.org/10.3889/oamjms.2021.6558)

Document Version

Publisher's PDF, also known as Version of record

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Citation for the published version (APA):

Iskandar, A., Lawanto, E. M., Zulhaidah, M., Olivianto, E., Handono, K., Anshory, M., Aprilia, A., & Aryati, A. (2021). The diagnostic value of urinary secretory antigen target of 6 kda in childhood pulmonary tuberculosis. *Open Access Macedonian Journal of Medical Sciences*, 9(B), 841-846. <https://doi.org/10.3889/oamjms.2021.6558>

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The Diagnostic Value of Urinary Secretory Antigen Target of 6 kDa in Childhood Pulmonary Tuberculosis

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Abstract

Edited by: Ksenija Bogojeva-Kostovska
Citation: Iskandar A, Lawanto EM, Zulhaidah M, Olivianto E, Handono K, Anshory M, Aprilia A, Aryati A. The Diagnostic Value of Urinary Secretory Antigen Target of 6 kDa in Childhood Pulmonary Tuberculosis. Open Access Maced J Med Sci. 2021 Aug 21; 9(B):841-846. https://doi.org/10.3889/oamjms.2021.6558
Keywords: Antigen; Tuberculosis; Childhood; Urinary early secretory target of 6 kDa antigen
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Received: 02-Jun-2021
Revised: 07-Aug-2021
Accepted: 11-Aug-2021
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Funding: This study was supported by the Ministry of Research and Higher Education of Indonesia and the Dean of Medical Faculty of Universitas Brawijaya
Competing Interests: The authors have declared that no competing interests exist
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BACKGROUND: Childhood tuberculosis (TB) is difficult to diagnosed and is based together on clinical and microbiology examinations. Since in children signs and symptoms of TB are not typical and sputum is difficult to be obtained, *Mycobacterium TB* (MTB) antigen detection could be considered as a non invasive method for early detection of childhood TB. Early secretory target of 6 kDa antigen (ESAT-6) is a low molecular weight specific protein that plays an important role in MTB virulence.

AIM: The aim of the study is to determine the diagnostic value of urinary ESAT-6 for the diagnosis of childhood TB.

METHODOLOGY: This was a cross-sectional study, with consecutive sampling collection. In children aging between 0 and 14 years suspected for pulmonary TB based on the clinical presence of: cough lasting more than 2 weeks, fever without clear etiology, loss of body weight or poor weight gain, fatigue, malaise with positive history of contact with sputum smear from adult TB patients. Diagnosis of pulmonary TB was based on clinical presentation plus positive tuberculin skin test, chest X-ray, acid-fast bacillus (AFB) staining, and/or sputum culture. Subjects who met the inclusion criteria but unconfirmed by clinical and microbiological were considered as control (non-TB group). Urinary ESAT-6 level was analyzed using ELISA. Cut-off value and area under the curve were determined using receiver operating characteristic (ROC) statistical analysis (SPSS 20.0). Sensitivity and specificity were measured from 2 × 2 crosstable.

RESULTS: Between the 61 studied children with suspected TB, 46/61 (75%) were finally diagnosed with TB, with 34/46 (74%) microbiologically confirmed cases either by sputum microscopy 31/34 (91%) or culture 3/34 (9%), whereas 15/61 (25%) subjects were not-confirmed cases (non-TB group). The mean value of urinary ESAT-6 level was higher in TB than non-TB group, Mean (SD) (4.855 [6.714]) ng/mL vs. (1.503 [0.946]) ng/mL; $p \leq 0.001$ (Mann-Whitney test). At ROC curve analysis, the cut off value of urinary ESAT-6 in subjects TB confirmed both with clinical plus microbiology evaluation as reference standard was 1.91 ng/mL, with sensitivity 72% and specificity 67%. While the cut-off value of ESAT-6 in TB subjects confirmed group only by clinical signs was 2.45 ng/mL, with sensitivity 65% and specificity 67%.

CONCLUSION: Urinary ESAT-6 has good diagnostic value and could be considered of value when used in addition to microbiological tests and clinical examination.

Introduction

Tuberculosis (TB) is one of the most prevalence infections worldwide and remained the most common cause of death from a single infection in 2019. The most prevalence region with TB infection is South-East Asia (44%) and the prevalence in Indonesia is 8.5% [1]. The worldwide TB case load in children is not completely widely known due to the lack of diagnostic tools for these subjects and inadequate system for recording and reporting cases of childhood TB. The diagnosis of childhood TB is very difficult, often inaccurate, and can result in over or underdiagnosis, leading as consequence to inadequate treatment [2]. In infants and children, the inability or difficulty to expectorate the sputum, the lower bacillary load than can cause false-negative results in staining (acid-fast stain) and culture [3]. Culture represents the gold standard examination of

adult TB diagnosis but with a poor sensitivity (30–40%) in children [4], [5]. Urine samples for diagnosis of TB have various advantages, especially in children, while it is a noninvasive tool that could be useful also in the case of extra-pulmonary TB and can be used in the contemporary presence of HIV infection which is characterized by fewer amount of bacteria in sputum [6]. Moreover, the WHO in 2011 banned the use of serological tests for the detection of antibodies against mycobacteria, since commercial serological tests provide inconsistent and imprecise findings for the detection of TB. Furthermore, the WHO also stated that until now there is no serology test viable that can be used for diagnosis of childhood TB and encourages further research in developing new TB serology tests for children based on antigen marker [7].

Early secretory target of 6 kDa antigen (ESAT-6) was known to play an important role in TB bacterial virulence. ESAT-6 can cause cytolysis of the membrane and thus the evasion of MTB inside

the phagosome [8]. ESAT-6 is a small protein for about 6 kDa and could be unrestrictedly filtered by the glomerulus and be detected in the urine [9]. The study about *in-vitro* assays that measure interferon- γ (IFN- γ) or IFN- γ secreting T cells indicate that ESAT-6 is a potential diagnostic reagent that highly specific for active TB and is often detected during early infection in TB disease [10].

Mukundan *et al.* (2012) demonstrated the direct detection of ESAT-6, measured by ELISA, in serum samples of patients with active TB showing that ESAT-6 was detected in three samples of active TB but not in non-TB control samples [11]. Other study by Song *et al.* (2014) examined the diagnostic tests of cerebrospinal ESAT-6 with indirect ELISA method on meningitis TB, obtained a sensitivity of 88% and specificity of 92% [12].

So far there is no data have been reported on the value of urinary ESAT-6 testing for diagnosis of TB both in adults and in children. The aim of our study was to evaluate the diagnostic value of urinary ESAT-6 in childhood TB.

Materials and Methods

Study design and patient population

Between June 2018 and June 2019, a cross-sectional study, with consecutive sampling collection, was conducted at Department of Child Health, Saiful

Anwar General Hospital in Malang, Indonesia. The study population was consisted of 78 children aging 0–14 years old with clinical suspicion of pulmonary TB. The study was approved by the ethical committee of the Faculty of Medicine Universitas Brawijaya/Saiful Anwar Hospital. Informed consent was obtained from either parent or relatives accompanying the patients.

Children aged 0–14 years suffered from cough more than 2 weeks, fever without clear etiology, loss of body weight or poor weight gain, fatigue, malaise, and have history of contact with positive sputum smear adult TB patient were enrolled into the study. Subjects previously diagnosed to have pulmonary or extrapulmonary TB, with symptoms and sign of extrapulmonary TB, such as chronic cervical, submandibular, and supraclavicular lymph node enlargement, spine angulation, joint swelling and had already received anti-TB medications for more than 2 weeks were excluded. Subjects were also excluded if there were leukocyturia, hematuria, and moderate-severe proteinuria reflecting renal abnormalities found in urinalysis.

History taking was also aimed to check if there was the presence of household adult contact with active TB and previous history of anti-TB medication. Physical examination was performed systematically including evaluation of nutritional status using maternal and child health book (Indonesian Ministry of Health) for children aged <5 years and the CDC 2000 curve for children aged >5 years [2]. Chest X-ray was interpreted by senior pediatric pulmonologist (MSC). Interpretation would be suggestive of TB or not. However, the result of chest X-ray would be considered in relation to overall clinical presentation. Tuberculin skin tests were performed in all subjects using 2 TU PPD RT-23, intradermally applied (Tuberculin skin test are considered positive if diameter of induration ≥ 10 mm) [13]. Urine sample (± 10 mL) was taken at the moment of subjects in hospital presentation and urinary ESAT-6 level was measured using the ELISA method (Bioassay Technology Laboratory) [14]. Sputum samples were obtained by sputum induction or gastric lavage while sputum examination for acid-fast staining (Ziehl-Neelsen) and culture (Lowenstein Jensen) was also performed.

Subjects were diagnosed as pulmonary TB and considered microbiologically confirmed when at least one positive result of sputum staining or positive MTB culture. If microbiology examination showed negative results, the subjects were considered as clinically confirmed TB when they defined as active TB by a pediatrician and has been designated to receive TB treatment and met at least two of the followings symptoms and signs suggestive of pulmonary TB; chest radiograph consistent with TB; close contact of TB or positive tuberculin skin test; and noted positive clinical response after 2 months course of anti TB treatment. Subjects who met the inclusion criteria but unconfirmed by clinical and microbiological were considered as non-TB group (Flowchart 1).

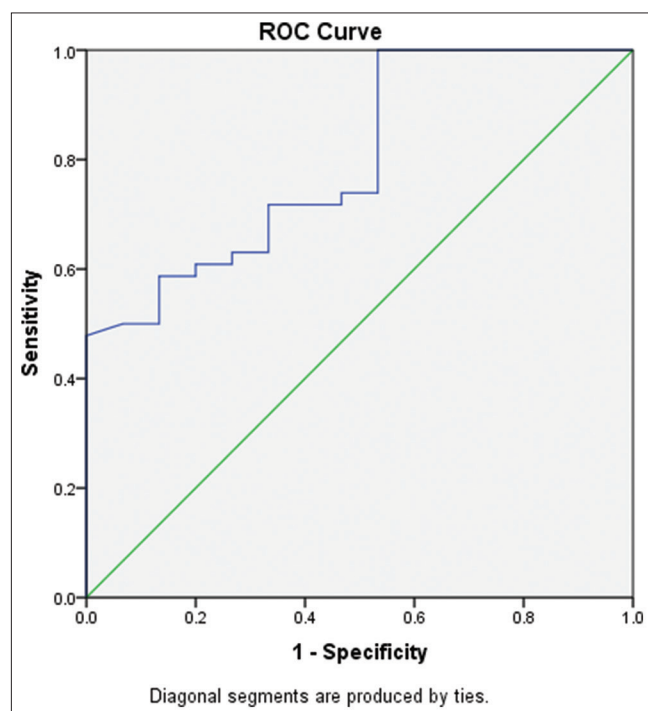
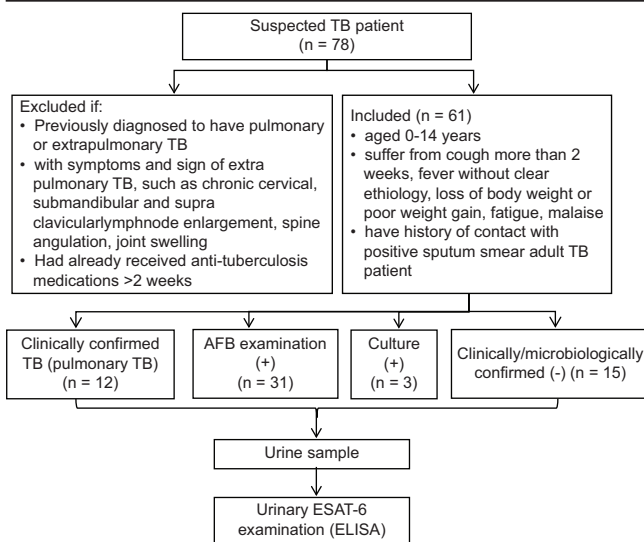


Figure 1: Area under the curve of this test was 0.8 (95%CI 0.68–0.92) by using receiver operating characteristic curve based on clinically diagnosed tuberculosis



Flowchart 1: The flowchart of the study

Statistical analysis

Sample size determined using the following formula [15]:

$$N = \frac{(Z_{\alpha} \sqrt{2V_1} + Z_{\beta} \sqrt{2V_1 + V_2})^2}{(AUC_1 - AUC_2)^2}$$

The urine ESAT-6 levels were evaluated as mean ± SD. To determine the differences in the levels of ESAT-6 in the 2 groups: (1) TB group and (2) non-TB group; the Mann-Whitney test or t-test were used with significance reached if p-value was at least <0.05. Receiver operating characteristic (ROC) curve plotted various intersection points for sensitivity and specificity values to determine the cut-off values. Positive predictive value (PPV), negative predictive value (NPV), positive likelihood ratio (PLR), and negative likelihood ratio (NLR) was calculated from 2 × 2 table analysis.

Results

Forty-six (75%) of 61 subjects suspected of TB were eventually diagnosed with TB and 15 (25%) were not. Of those diagnosed with TB, 34 (74 %) were microbiologically confirmed cases either by sputum microscopy (91%) or culture (9%), and 12 (26%) subjects were clinically confirmed TB. The characteristics of the subjects were shown in Table 1.

Urinary ESAT-6 level was higher in subjects with TB (4.855 ± 6.714) ng/mL compared to non-TB group (1.503 ± 0.946) ng/mL and statistically significant using Mann-Whitney test (p = 0.001). Using SPSS 20.0 software, cut-off value of urinary ESAT-6 level

Table 1: Characteristics of subjects

Characteristics	Non-TB (n = 15)	TB (n = 46)
Age (year)	2.04 ± 2.91	6.98 ± 4.32
0-1	10 (66.7)	2 (4.3)
>1-5	3 (20.0)	16 (34.8)
>5-14	2 (13.3)	28 (60.9)
Male sex	10 (66.7)	30 (65.2)
Positive Tuberculin skin test	0	26 (56.5)
Positive microbiology test	0	34 (73.9)
Kultur (+)	0	3 (6.5)
BTA (+)	0	31 (67.4)
Urinary LAM (ng/mL)	1.503 ± 0.946	4.855 ± 6.714
Nutritional status		
Normal	7 (46.7)	18 (39.1)
Malnourished	8 (53.3)	28 (60.9)

Data was presented in n (%), and mean ± SD, TB: Tuberculosis.

was 1.91ng/mL when compared to diagnosis of TB (including clinically and microbiology confirmed cases). When only positivity of microbiology examination used as standard reference, cut off value of urinary ESAT-6 level was 2.45 ng/mL.

Table 2 showed the number of subjects based on the value of the cut-off ESAT-6 and diagnosis of TB. Based on this 2 × 2 crosstable, the sensitivity of the test was 72%, specificity 67%, PPV 87%, NPV 43%, PLR 2.18, and NLR 0.42.

Table 2: Urinary ESAT-6 level (cut off point 1.91 ng/mL) and diagnosis of TB 2 × 2crosstable

Urinary ESAT-6	Diagnosis		Total
	TB	Non-TB	
ESAT-6 ≥1.91 ng/mL	33	5	38
ESAT-6 <1.91 ng/mL	13	10	23
Total	46	15	61

ESAT: Early secretory target of 6 kDa antigen, TB: Tuberculosis.

Table 3 showed the number of subjects based on the value of the cut-off ESAT-6 and microbiological confirmed (acid-fast staining or culture). Based on this 2 × 2 crosstable, the sensitivity of the test was 65%, specificity 67%, PPV 71%, NPV 60%, PLR 1.97, and NLR 0.52.

Table 3: Urinary ESAT-6 level (cut off point 2.45 ng/mL) and microbiological confirmed 2 × 2 crosstable

Urinary ESAT-6	Microbiological confirmed		Total
	Acid fast stain/culture (+)	Acid fast stain/culture (-)	
ESAT-6 ≥2.45ng/mL	22	9	31
ESAT-6 <2.45ng/mL	12	18	30
Total	34	27	61

ESAT: Early secretory target of 6 kDa antigen.

ROC curve analysis showed area under the curves (AUCs) of 0.8 (95% CI 0.68–0.92) to the clinical diagnosis of childhood TB (Figure 1), and when we analyze by using microbiological confirmation as reference standard, the AUCs was 0.71 (95%CI 0.58-0.84) as shown in Figure 2.

Discussion

In order to provide a definitive diagnosis of TB is mandatory to find MTB on microbiological examination, including direct microscopic examination of smear or tissue biopsy and culture examination. However, the

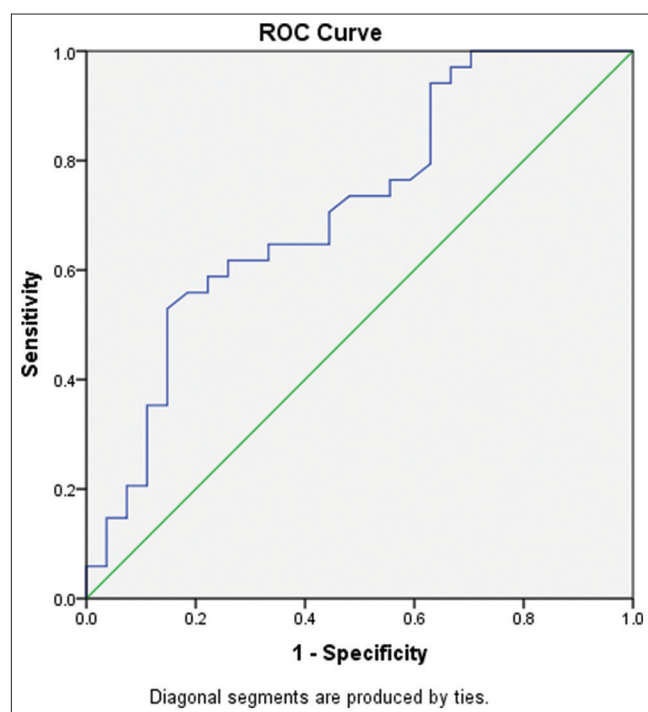


Figure 2: Receiver operating characteristic curve showed area under the curve of this test was 0.71 (95%CI 0.58–0.84) by using microbiological confirmed as reference standard

diagnosis of childhood TB presents many caveats, such as (1) TB can mimic many common childhood diseases, including pneumonia, generalized bacterial and viral infections, (2) the absence of a practical reference test or gold standard, (3) the inability of pediatric patients to provide sputum, (4) A nonspecific clinical presentation (up to 50% of children may be asymptomatic in early stages of the disease), (5) the lower bacillary load often smear negative [16]. The number of MTB in the bronchial secretions of children is fewer than adults because of the location of tissue damage in primary pulmonary is located in the hilar lymph nodes and peripheral lung parenchyma and the severity of lung damage is not as severe as in adult patients [3]. (6) Confirmation by culture of MTB, the gold standard of diagnosis in adult TB, rarely exceeds 30–40% sensitivity.

As consequence the diagnosis of childhood TB can be established only by coupling clinical symptoms and laboratory tests [16].

In this study, the levels of urinary ESAT-6 were significantly different between TB and non-TB groups, either by microbiological and/or clinically confirmed as well as microbiological confirmation only. The study by Mukundan *et al.* in 2012 is the first study showing the direct detection of ESAT-6 in serum samples obtained from patients with active TB. In these studies, examination of ESAT-6 (ELISA) conducted on 6 plasma samples in which three samples were samples obtained from active TB and 3 other samples were non-TB control and the results were obtained that ESAT-6 was detected in three samples of active TB but not in non-TB control samples [11]. The study

by Song *et al.* (2014) was a diagnostic test ESAT-6 (indirect ELISA) in meningitis TB, obtained sensitivity of 88% and a specificity of 92%, there are significant differences between the mean levels of cerebrospinal fluid ESAT-6 in patients with meningitis TB compare with non-meningitis TB. The ESAT-6 level was higher in meningitis TB than non-meningitis TB [12].

The sensitivity and specificity of urinary ESAT-6 based on only microbiological confirmed was lower than the sensitivity and specificity urinary ESAT-6 based on microbiological and/or clinically confirmed. It may be caused by the difficulties in sampling of the children and paucibacillar conditions in children. In these cases, the diagnosis of TB can not use microbiologic examination only but also required clinical confirmation. This is supported by research Triasih and Graham (2011), which shows that the positive results of microbiological examination of sputum are only found in 10–15% of patients with suspected TB [5], and according to research by Jahromi (2014) positive cultures were obtained in 30–40% of cases [4]. Jahromi and Mood (2014) showed that sensitivity of culture examination is low in children because its general form is paucibacillary, difficult to obtain sputum samples for children who can not cough up their sputum [4]. To overcome this, the selected samples from other locations were used such as culture of morning gastric lavage sample had a sensitivity of 30–40% while the nasopharyngeal aspiration was 24–30%. Negative culture results cannot rule out the possibility of childhood TB. Other microscopic examination whose values were equal to culture examination on childhood TB is sputum smear examination, this examination has a sensitivity that varies between 25% and 75%, but in children, sensitivity is only <15%. Most children with active TB are negative in smear examination and 10–15% of the children were shown to have smear-positive TB. Based on the data from the Indonesian Ministry of Health for TB Program in 2011, the percentage of smear-positive TB cases in children 0–14 years is 6.3% of all TB cases children, this figure increased from 2010 at 5.3% [2].

Until now, there were no studies that have been published regarding the diagnostic values of urinary ESAT-6 TB patient, especially in children. Urine samples have various advantages, especially in child whose sputum was paucibacillar, difficulty to expectorate the sputum, less invasive than blood or biopsy samples in extrapulmonary TB cases and can be used in cases of TB with HIV, whose sputum generally have fewer bacteria than in TB without HIV [6]. TB diagnosis in children has many flaws though using the gold standard. Until now, Indonesia using the TB scoring system-IDA1 to diagnose TB in children. In other hand, there have been several studies that claim that this scoring system could cause inaccuracy TB diagnosis problems especially if there is history of household contact with TB patient. The study of Triasih and Graham state that the scoring system had a sensitivity of 47% and

specificity of 68%. These results indicate that the usage of TB scoring system to diagnose childhood TB need to be reobserved [5].

The TB diagnosis in this study did not use TB scoring system as defined by IDAI since 2008 but done by combining the clinical signs and symptoms and TB smear or culture. So that, the gold standard in this study more accurately described as reference standard. The reference standard used in this study was microbiological confirmed and/or clinically confirmed by competent pediatrician [17], [18].

ESAT-6 is a small size, 6 kDa protein and appears as a heterodimer in culture supernatants. ESAT-6 encoded by the region of difference 1 (RD1) of MTB which is an area that has consistently not found in attenuated or avirulen *Mycobacterium bovis* strains BCG. MTB uses the ESX-1 secretion system to deliver virulence proteins including ESAT-6 during infection of host cells. ESAT-6 forms a complex with culture filtrate protein-10 kDa (CFP-10) as a 1:1 ESAT-6:CFP-10 complex before exported out of the cell, ESAT-6 can be separated from her partner CFP-10 at acidic pH (such as inside the phagosome). Individually, ESAT-6 may cause membrane disruption and cytolysis [7]. ESAT-6 that is apart from CFP-10 will binds to liposomes containing dimyristoyl phosphatidylcholine and cholesterol (a component of mammals' cell membranes), causing instability and lysis of the liposomes. So that, ESAT-6 also plays a role in MTB mechanism to avoid the phagosome [19].

ESAT-6 size is small enough to allow its filtered unrestrictedly by the glomerulus so that can be detected in the urine. Kashino *et al.* in 2008 conducted a study of patients with active pulmonary TB which proved positive MTB culture patient after rule out any abnormalities or disorders of kidney and urinary tract as well as the possibility of renal TB. In such patients, TB antigen were detected in their urine which indicates that the TB antigens are derived from TB infection in the lungs [20].

Study limitations

The limitation of this study were the urine sample in this study were performed with random urine with various concentration of its analytes within it and it was difficult to obtained urine sample in pediatric patients, especially in child using diapers. Beside those, we did not examine urine of healthy control without TB.

Conclusion

We conclude that the levels of ESAT-6 urine were higher in groups of patients with TB than non-TB.

The diagnostic value of urinary ESAT-6 is moderate with sensitivity of 72% and specificity of 67%. Urinary ESAT-6 has good diagnostic value and could be considered of value when used in addition to microbiological tests and clinical examination.

Acknowledgment

We would like to thank to our colleagues from Department of Childhood, Faculty of Medicine Universitas Brawijaya/Saiful Anwar General Hospital, for the warmest cooperation. We also thank to Ministry of Research and Higher Education of Indonesia, and Dean of Medical Faculty of Universitas Brawijaya for supporting funding of the research.

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