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 virulence. ESAT-6 can cause cytolysis of the membrane and thus the evasion of MTB inside bacterial virulence. ESAT-6 is known to play an important role in 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.

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

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Abstract

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 33/34 (91%), 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 ≤ 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 evalution as reference standard was 1.91 ng/mL, with sensitivity 72% and specificity 67%. While 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%.

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

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.
**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 was 1.91 ng/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 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 72%, PPV 87%, NPV 43%, PLR 1.91 ng/mL when compared to diagnosis of TB.

**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...
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 paucibacillary 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, difficulty 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 paucibacillary, 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-IDAI 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
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 avirulent 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.

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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