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# High expression of early secretory antigenic target 6 mRNA as a potential predictor of tuberculous lymphadenitis



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## ABSTRACT

**Purpose:** Tuberculous lymphadenitis is one of the most common presentations of extrapulmonary tuberculosis. The diagnosis can be challenging due to its varying clinical manifestations and the low sensitivity of conventional bacteriological methods for confirming the causative agent, *Mycobacterium tuberculosis*. Early secretory antigenic target 6 (ESAT-6), which is released early during *M. tuberculosis* infection and plays a role in granuloma formation, determines the pathogen's invasion, its severity, and virulence factors related to the immune response. This study aimed to analyze the potential diagnostic value of ESAT-6 using real-time polymerase chain reaction (RT-PCR) and compare it with histopathologic diagnostics.

**Materials and methods:** This retrospective observational study with the cross-sectional design was conducted using consecutive sampling. A total of 50 formalin-fixed, paraffin-embedded lymph node tissue samples from lymphadenectomy consisted of 25 tuberculous lymphadenitis and 25 granulomatous lymphadenitis

were collected. The specimens were diagnosed with tuberculous and granulomatous lymphadenitis based on histopathologic features. Analyses using conventional PCR from commercial kit to detect TB and RT-PCR to determine the expression of ESAT-6 were performed. Statistical analysis of the categorical data was performed using the Mann-Whitney test. Statistical significance was set at  $p < 0.05$ .

**Results:** The mean age of the patients was 35.66 years old (range: 6–85 years). The age group between 16 and 30 years was the most common (40%). Almost two-thirds (62%) of the patients were female. Sixteen (64%) tuberculous lymphadenitis and 13 (52%) granulomatous lymphadenitis samples were positive by PCR TB. ESAT-6 expression was significantly higher in tuberculous lymphadenitis ( $p = 0.004$ ).

**Conclusion:** ESAT-6 as a potential predictor of tuberculous lymphadenitis can be used in patients for whom histopathologic diagnostics fail to confirm the presence of *M. tuberculosis*.

**Keywords:** tuberculous lymphadenitis, real-time PCR, ESAT-6

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## INTRODUCTION

*Mycobacterium tuberculosis* remains a leading cause of death in humans. In 2018, the incidence of TB deaths among HIV-negative patients was reported to be about 1.2 million. Indonesia ranked third in the world in terms of tuberculosis incidence after India and China.<sup>1</sup> Tuberculous lymphadenitis is the most common manifestation of extrapulmonary tuberculosis. In Indonesia, it accounts for over 60% of all extrapulmonary tuberculosis cases, according to several hospital reports.<sup>2</sup>

Due to its varying clinical manifestations, the conventional clinical diagnosis of tuberculous lymphadenitis can be indefinite. Fine needle aspiration cytology is usually the first line of investigation and can be confirmed with Ziehl-Neelsen staining for acid-fast bacilli, mycobacterial cultures, and nucleic acid amplification tests. Culture identification is the gold standard for

diagnosis, but it may take two to four weeks to yield results. A positive acid-fast bacillus stain is widely used. However, its variability and generally low sensitivity can obscure the distinction from nontuberculous mycobacteria.<sup>3</sup>

Diagnosis of tuberculous lymphadenitis from tissue samples is usually established by histopathologic examination. Histological features, such as epithelioid cell granulomas and Langhans cells with central caseous necrosis, support the diagnosis; however, not every case presents with caseous necrosis.<sup>4</sup> Other causes of chronic lymphadenitis include infectious and noninfectious granulomatous disorders.<sup>3</sup> None of these methods alone can diagnose all cases of tuberculous lymphadenitis. A fast and cost-effective technique to reliably diagnose tuberculous lymphadenitis is, therefore needed, particularly in resource-poor settings.

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Early secretory antigenic target 6 (ESAT-6) is a protein encoded by the *RD1* gene that is consistently absent from all avirulent strains of *M. tuberculosis*. It is secreted from the bacterium during growth in the early phase of infection.<sup>5</sup> This protein can interact with various cells that modulate the host's immune response and prevent phagosome maturation and transmigration of bacteria from phagosomes to cytosols.<sup>6</sup> It also plays a role in granuloma formation and tissue necrosis and determines the severity of disease,<sup>7</sup> tissue differentiation via matrix metalloproteinase,<sup>8</sup> and development of caseous granuloma.<sup>9</sup> Sequence IS6110 usually appears 6–20 times in the *M. tuberculosis* complex genome<sup>10</sup> and has higher sensitivity rates for DNA amplification of formalin-fixed, paraffin-embedded tissues compared to other targets, such as MTP40 and 65-kDa antigens.<sup>11</sup> A measurement of ESAT-6 expression in tissue specimens to diagnose tuberculosis has not been performed previously. This study aimed to analyze the potential diagnostic value of ESAT-6 using real-time polymerase chain reaction (RT-PCR) and compare it with histopathologic diagnostics. In addition, *M. tuberculosis* involvement is also confirmed using commercial PCR kit targeting IS6110.

## MATERIALS AND METHODS

This was a retrospective observational study with a cross-sectional design using consecutive sampling. This study initially collected 48 formalin-fixed, paraffin-embedded lymph nodes with histopathological diagnosis of tuberculous lymphadenitis during the sampling period from 2016 to 2017. The samples were obtained from Cito Laboratory, Yogyakarta, Indonesia. Of those samples, only lymph nodes removed in toto with minimal necrosis, good tissue and paraffin block quality were included in this study. Biopsy samples, incomplete clinical data and samples from patients with immunocompromised diseases such as HIV were excluded in this study. In the end, 25 samples of tuberculous lymphadenitis fulfilling the criteria were included. Additional 25 lymph nodes diagnosed with granulomatous lymphadenitis with similar clinical data were collected as comparison. Histopathological preparations were reviewed and determined by two pathologists separately, with Kappa score of 1. All specimens were collected with the approval of the ethical committee at the Medical and Health Research Ethics Committee (MHREC) Faculty of Medicine, Public Health, and Nursing Universitas Gadjah Mada – Dr. Sardjito General Hospital (Ref. No.: KE/FK/0611/EC/2017).

Mycobacterial genomic DNA was extracted from the samples using total DNA (QIAGEN) according

to the manufacturer's instructions. PCR was carried out using Bioneer Exicycler™96 Real-Time Quantitative Thermal Block. The PCR condition was as the manufacturer's recommendations. Positive control DNA standards of confirmed tuberculosis patients and negative controls (sterile double-distilled water) were used in each run. If the cycle threshold (Ct) value of the sample is  $\geq 40$ , the result of the assay was classified as negative. If the Ct value of the sample was  $< 40$ , the test result was classified as positive.

RNA was extracted using total RNA kit (QIAGEN Cat No./ID: 73604) according to the manufacturer's instructions. RT-PCR amplification was performed using the *ESAT-6* gene with forward primer 5'-TCC ATT CAT TCC CTC CTT GA-3', reverse primer 5'-TTT GCT TGG ACA CCC TGG TA-3', and *GAPDH* as a reference gene with forward primer 5'-GCA TCC TGG GCT ACA CTG AG-3' and reverse primer 5'-TCC ACC CTG TTG CTG TA-3'. Quantitative PCR analysis for RNA was conducted in the same manner as the DNA protocol. The condition of the PCR was as recommended by the manufacturer.

The sample size was based on the consecutive sampling of samples available that year. The obtained data were analyzed to compare the mean expression value of *ESAT-6* with the group of histopathologic diagnosis and tuberculosis status. Statistical analysis was performed using IBM SPSS Statistics version 20 (IBM Corp.). The normality of data distribution was assessed using the Shapiro-Wilk test and Levene's test. As the data distribution was not normal, the Mann-Whitney test was used. Categorical data were expressed as frequency and percentage, and quantitative data were expressed as means  $\pm$  SD. Statistical significance was set at  $p < 0.05$ .

## RESULTS

During the sampling period, 73 samples were initially collected. Based on the inclusion and exclusion criteria, 50 samples were included in the analysis. The mean age of the patients was  $35.66 \pm 18.88$  years (range: 6–85 years). The age group of 16–30 years was the most common (40%). Almost two-thirds (62%) of the patients were female (Table 1). The expression levels of *ESAT-6* between tuberculous lymphadenitis and granulomatous lymphadenitis were significantly different ( $p = 0.004$ ) (Table 2).

## DISCUSSION

Resources are limited in developing countries, particularly for diagnostic procedures that

**Table 1. Characteristics of the study samples**

Characteristic	Tuberculous lymphadenitis n = 25	Granulomatous lymphadenitis n = 25	Total n = 50
<b>Sex</b>			
Female (%)	15 (60)	16 (64)	31 (62)
Male (%)	10 (40)	9 (36)	19 (38)
<b>Age</b>			
<15 (%)	3 (12)	1 (1)	4 (8)
16–30 (%)	11 (44)	9 (10)	20 (40)
31–45 (%)	7 (28)	5 (20)	12 (24)
46–60 (%)	2 (8)	6 (24)	8 (16)
>60 (%)	2 (8)	4 (16)	6 (12)

**Table 2. Lymphadenitis status across ESAT-6 mRNA and TB kit detection**

Characteristics	Tuberculous lymphadenitis	Granulomatous lymphadenitis	<i>p</i>
ESAT-6 mRNA			
Mean ± SD	14.39 ± 8.19	8.45 ± 7.09	0.004*
TB Kit			
Positive	16 (64%)	13 (52%)	
Negative	9 (36%)	12 (48%)	

Notes: \*Mann-Whitney test.

Abbreviations: SD, standard deviation.

include lymph node biopsy. In our study, 9 cases of histopathologically reported tubercular lymphadenitis were negative PCR TB. The number of IS6110 copies varies significantly between *M. tuberculosis* strains. In Southeast Asia, some strains (8–11%) lack the IS6110 gene (no-copy strains).<sup>12</sup> This may cause negative results in our study. In addition, the negative result can be contributed by the existence of inhibitors that reduce the performance of PCR amplification in the molecular diagnosis of TB.<sup>13</sup> Inhibitors involving blood, protein, and eukaryotic DNA can be found in clinical specimens such as biopsy and pleural effusion. Non-uniform distribution of bacillus bacteria in aliquot for diagnosis can also cause false-negative outcomes.<sup>13</sup> This has shown that the molecular diagnostic approach has a high sensitivity in positive bacteria samples but is less sensitive in paucibacillary samples.<sup>14</sup> Therefore, the negative diagnostic results in our study still cannot rule out tuberculosis infection.

The PCR TB assay, in addition, also showed positive result in 13 (52%) granulomatous lymphadenitis samples. This may be caused by inadequate features of the specimen to be histopathologically diagnosed as tuberculous lymphadenitis. Histological features, such as epithelioid cell granulomas and Langhans cells with central caseous necrosis, may support the diagnosis

of probable tuberculosis; however, caseous necrosis is not present in every case.<sup>4</sup> This may result in misdiagnosis of the sample histopathologically.

PCR can facilitate early and accurate detection of tuberculosis-causing organisms with high sensitivity and rapidity. PCR is useful for the diagnosis, treatment, prevention and control of this disease. The traditional method of assessment of tuberculosis takes 4–8 weeks for diagnosis.<sup>15</sup> Living bacteria are needed for culture, but only the DNA genome is needed to detect tuberculosis by PCR.<sup>16</sup> mRNA detection by RT-PCR, however, has been recommended for the detection and quantification of *M. tuberculosis* with more rapid results, lower risk of contamination, facilitation of nucleic acid quantification, and automation and computerization of data.<sup>17</sup> RT-PCR can detect cases that are missed by conventional PCR in cerebrospinal fluid samples.<sup>18</sup> Adding multiple sites of the *M. tuberculosis* genome (IS6110, MPB64, and PT8/9) using conventional PCR does not increase the positivity rate.<sup>19</sup> DNA PCR cannot differentiate between viable and nonviable organisms. Bacterial mRNA, with a mean half-life of 3–5 min, is more prone to destruction than genomic DNA. This unique characteristic showed that positive mRNA expression could be more accurate in indicating the presence of viable organisms.<sup>20–21</sup>

Our study found significantly upregulated ESAT-6 expression in 25 samples histopathologically diagnosed with tuberculous lymphadenitis ( $p = 0.004$ ). No previous studies have compared ESAT-6 expression levels in tuberculous and granulomatous lymphadenitis patients. One study used type II alveolar epithelial cells (AEC) to determine the ESAT-6 expression of *M. tuberculosis* and found significantly elevated levels compared to a negative control in infection with strain H37RV.<sup>22</sup> Our study showed that ESAT-6 expression was another ancillary test for diagnosing TB and can be used to diagnose tuberculosis in unconfirmed lymphadenitis.

Our study showed that tuberculous lymphadenitis affects more females (~60%) than males. Other studies have reported similar percentages.<sup>23–25</sup> Biologically, there is a fundamental difference between males and females' hormonal and immune systems. Females usually have a quiescent presentation of symptoms.<sup>23</sup> Socially, their low socioeconomic and nutritional status in developing countries can affect their immune response to the disease.<sup>10</sup> Moreover, females in those countries tend to stay or work at home.<sup>25</sup> This condition with less air ventilation may increase the risk of tuberculosis progression.<sup>25</sup> It has also been shown that women appear to be more attentive to

health and attend health facilities earlier than males when symptoms are present.<sup>23</sup> More female patients reported in the data for health care facilities may result from this analysis.

In this study, a significant proportion of patients with tuberculous lymphadenitis (44%) belonged to the age group of 16–30 years. Kamal et al. similarly reported a high percentage (61.5%) in this age group.<sup>25</sup> Study by Purohit et al. also showed similar findings with about 75% of the patients were aged between 14 and 35 years old.<sup>23</sup> These findings indicate a change in tuberculosis's epidemiology, with a shift in the peak prevalence of tuberculous lymphadenitis from adolescents to the ages of 20–40 years.<sup>23</sup> Our results are consistent with this shift.

We reported that ESAT-6 expression by RT-PCR in lymphadenitis can be used to predict *M. tuberculosis* involvement. Despite the findings, there were few limitations to this study. First, we did not identify the strain of *M. tuberculosis* in our study. The difference in *M. tuberculosis* strains may result in different expressions that may affect our study results. Second, each cut of the lymph nodes section may have different granuloma appearance. This may result in variations in the amount of *M. tuberculosis* DNA between samples.

## CONCLUSION

The *ESAT-6* mRNA was significantly upregulated in samples with histopathologically diagnosed tuberculous lymphadenitis. In the failure of histopathological examination to confirm the involvement of *M. tuberculosis*, the *ESAT-6* may serve as a potential predictor of tuberculous lymphadenitis.

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## AUTHOR CONTRIBUTION

All authors have contributed to all process in this research, including research design, data collection, and its analysis, writing the manuscript for article publication.

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## CONFLICT OF INTEREST

The authors declare no conflict of interest regarding the publication of this article.

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