

SEVERITY OF TB CLASSIFIED BY MODIFIED BANDIM TB SCORING ASSOCIATES WITH THE SPECIFIC SEQUENCE OF ESXA GENES IN MDR-TB PATIENTS

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Abstract

Background: The severity of pulmonary TB and detection of multidrug-resistant (MDR-TB) TB strains as potential causative agents could be crucial for the determination of treatment success. This study aimed to analyze the association between the specific sequences of the full *esxA* gene from MDR-TB sputum isolates and the severity class of MDR-TB patients.

Material and Methods: A total of 98 sputum samples that were suspected to be MDR-TB were collected from Dr. Soetomo, Surabaya, Indonesia, from September to December 2016. A total of 24 isolates from the 98 patients were confirmed to have positive MDR-TB based on the GeneXpert test. MDR-TB isolates were tested using PCR targeting 580 bp encompassing the full *esxA* gene, and the resulting amplicon was sequenced. The severity class of the pulmonary TB patients was assessed using modified Bandim TB scoring.

Results: The patient severity classification resulted in a moderate and severe degree of TB in 38% and a mild degree of TB in 63% of patients. Visualization of the PCR results showed that all MDR-TB samples were positive for the 580 bp band, and the sequence results showed 100% homology with that of the virulent wild-type *M. tuberculosis* H37Rv (NC_000962.3).

Conclusions: In the current study, an association between the characteristics of the full *esxA* gene and the severity class of MDR-TB patients is yet to be found. However, the homologous sequence of all samples, associated with various degrees of disease severity, possess 100% identity with that of wild-type *M. tuberculosis* H37Rv.

Keywords: Modified Bandim TB scoring; severity of TB disease; full sequence of *esxA* gene.

List of Abbreviations: Anti-TB drugs - Anti-tuberculosis drugs, BCG - Bacillus Calmette-Guerin, CFP-10 - 10-kDa culture filtrate protein, DNA - deoxyribonucleic acid, ESAT-6 - 6 kDa early secretory antigenic target, *M. tuberculosis* - *Mycobacterium tuberculosis*, MDR-TB - Multidrug-resistant tuberculosis, MTBC - *Mycobacterium tuberculosis* complex, MUAC - mid upper arm circumference, NO - nitric oxide, PCR - polymerase chain reaction, RD-1 - region of difference 1, ROS - reactive oxygen species, RR-TB - rifampicin-resistant tuberculosis, SC - severity class, TB - Tuberculosis, WHO - world health organization, XDR-TB - extensively drug-resistant tuberculosis

Introduction

Multidrug-resistant tuberculosis (MDR-TB) is one of the currently rising diseases with a high number of deaths. The world health organization (WHO) reported that pulmonary rifampicin-resistant tuberculosis (RR-TB) is a case in which *Mycobacterium tuberculosis* (*M. tuberculosis*) becomes resistant towards rifampicin, one of the strongest TB medicines, but is not always resistant to other anti-tuberculosis drugs (anti-TB drugs). The WHO accounted for 10,4 million TB cases worldwide, with mortalities as high as 1,4 million in 2015 (WHO, 2016). India, Indonesia, and China are the top three countries with the highest case number among all the countries in the world, with percentages of 23, 10, and 10% of the worldwide incidence of TB, respectively (WHO, 2015). In addition, 480,000 new cases of MDR-TB occurred globally in 2015 with detection and notification of 132,120 cases of MDR-TB or rifampicin-resistant TB (RR-TB). Even worse, 100,000 people infected by RR-TB needed second-line treatment (WHO, 2016).

Indonesia was ranked as the second highest country for TB burden in the world in 2014 and 2015. New cases of MDR-/RR-TB in Indonesia in 2015 accounted for 2,8% of the total 32,000 MDR-/RR-TB incidence. The incidence rate of all TB cases in Indonesia in 2015 was approximately 395 cases per 100,000 population, while the estimated incidence of MDR-/RR-TB cases among notified pulmonary TB in 2015 was approximately 10,000 cases (WHO, 2015; WHO, 2016). Data from the Ministry of Health in Indonesia (Indonesian Ministry of Health, 2016) also showed a total of 1,860 confirmed MDR-/RR-TB cases out of 15,380 suspected patients in Indonesia in 2015, with 1,566 patients treated.

RR-TB has become the main threat in TB management globally because a high number of RR-TB infections have evolved to be MDR-TB. Only 52% of MDR-TB patients were being treated successfully, mostly because of a high rate of mortality and patients lost to follow up (WHO, 2016). Mutations causing bacteria to be resistant to antibiotics are always correlated with a fitness cost to the bacteria (Cohen et al., 2003; Bhunia et al., 2015; Melnyk et al., 2015). An earlier study revealed that resistance being caused by mutations could impair the fitness of bacteria because mutations targeted genes encoding important cellular functions (Melnyk et al., 2015).

Another study also stated that mutations resulting in antibiotic resistance from in vitro research could decrease the growth rate and virulence of bacteria. In addition, resistant *M. tuberculosis* was found to be less viable than antibiotic-sensitive *M. tuberculosis* both in vivo and in vitro. Several cases have shown that resistant *M. tuberculosis* also has a lower transmission rate (Kim et al., 2006; Salvatore et al., 2016). Previous study also informed that mutants with both lower and equal fitness compared to antibiotic-sensitive *M. tuberculosis* had been found (Cohen et al., 2003).

In a study sequencing the whole genome of rifampicin-resistant *M. tuberculosis* isolates, it was suggested that within the context of rifampicin resistance, the frequency of clinical mutations could be an indirect measurement of the in vivo fitness of resistant *M. tuberculosis* strains among patients with pulmonary RR-TB, which was found to be varied (Comas et al., 2012). According to those studies, it is suspected that mutations in genes being involved in anti-TB drugs, such as *rpoB*, *katG*, and other genes, can affect the secretion of virulence genes, generating different degrees of severity to manifest in RR-TB patients.

The *esxA* gene, which encodes a 6 kDa early secretory antigenic target (ESAT-6), is an important virulence factor of *M. tuberculosis*. The *esxA* gene belongs to region of difference 1 (RD-1). The RD-1 region is removed to create the BCG (Bacillus Calmette-Guerin) vaccine. ESAT-6 is known to hinder macrophage activation, induce apoptosis, and disturb with host immunity. ESAT-6 is also a membrane lytic factor that let *M. tuberculosis* to avoid phagosome attack because ESAT-6 is able to suppress autophagosome formation. Another potential role of ESAT-6 and 10-kDa culture filtrate protein (CFP-10) is in activating an inflammation reaction (Yu and Xie, 2012; Solans et al., 2014).

The virulence of *M. tuberculosis* can induce different degrees of disease severity on each host. Different manifestations of tuberculosis illustrate the balance between infecting pathogens and host immune mechanisms (Shanmuganathan and Subramaniam, 2015). The degree of disease severity in patients can be measured using several methods, including the Bandim TB score. The Bandim TB score is a score based on the six signs and five symptoms indicated by the patient. The disease severity of TB has previously been shown to correlate with the bacterial load obtained from positive cultures and the response of patients to treatment, as shown by culture conversion rate at 2 months. The development of methods to classify the disease severity of TB could improve clinical care, preventive therapy, TB diagnostic methods, TB treatment, and vaccine trials (Wiseman et al., 2012; Rudolf et al., 2013).

The goal of this study was to analyze the association between TB disease severity based on Bandim TB criteria and specific sequences of the full *esxA* gene from sputum isolates of MDR-TB patients. Sequence analysis of the full *esxA* gene is essential to provide insight into the role of *esxA* genes in the infection rate of pulmonary RR-TB. This study was able to provide scientific information regarding the characterization of nucleotide sequences of the 580 bp *esxA* gene from isolates of MDR-TB patients.

Materials and Methods

A total of 98 sputum samples of suspected MDR-TB patients from Dr. Soetomo Academic Hospital, Surabaya, Indonesia, were collected from September to December 2016. The total samples were gained by Lemeshow *et al.* (1990) formula with estimation of TB patients proportion counted as much as 50% and estimation of healthy people proportion were 50%. The samples were collected by consecutive sampling. Among 98 patients, 24 isolates were confirmed to be positive for rifampicin resistance based on the GeneXpert test (Cepheid, 2015). Rifampicin resistance-positive samples were then tested by using polymerase chain reaction (PCR) performed at the TB Laboratory, Institute of Tropical Disease, Universitas Airlangga, Surabaya. The study was approved by the ethics committee in health research of Dr. Soetomo Academic Hospital with ethical clearance number 541/Panke.KKE/IX/2016.

Sputum samples were first decontaminated before being extracted using the Alkali Petroff's method recommended by the WHO (WHO, 1998; Parija, 2012). DNA was then extracted using a QIAamp DNA Kit (DNeasy Blood & Easy Kit, Cat No. 69504). The primers used in the current study were *esxA* Full Forward 5'-GCAATATTCGTCAGGCCGGCGTCCAATACT-3' and *esxA* Full Reverse 5'-CGTGCCATATCGTCCGGAGCTTCCAT-3'. The primers were designed using Clone Manager software 6, version 6.00 targeted at 580 bp. The KAPA2G Fast ReadyMix PCR Kit (KAPA Biosystem, USA) was used as a PCR Master Mix, containing DNA polymerase enzyme, buffer with dye, MgCl₂, and dNTPs. The solution mixture consisted of 25

µl of KAPA2G Fast ReadyMix PCR Kit, 1 µl of 10 µM primer pair, 20 µl of nuclease-free water, and 3 µl of DNA template.

The amplification reaction in the thermal cycler was initiated by predenaturation at 95°C for 3 minutes, followed by denaturation at 95°C for 10 seconds, annealing at 58.3°C for 10 seconds, and extension at 72°C for 15 seconds. Denaturation, annealing and extension were conducted for 35 cycles and finished with a final extension at 72°C for 10 minutes. Positive results were confirmed by the existence of DNA bands at 580 bp. PCR products were sent to 1st BASE, Singapore. 1st BASE used an ABI PRISM 3730xl Genetic Analyzer by Applied Biosystem. The sequence of the samples was analyzed using the BioEdit program version 7.2.5 (Ibis Therapeutics, USA) and NCBI BLAST to determine the homology percentage between the sample sequence and the wild-type *M. tuberculosis* H37Rv (NC_000962.3) sequence referenced from GenBank.

The severity class of pulmonary MDR-TB patients was assessed using modified Bandim TB scoring based on criteria of clinical manifestation disease by examining five symptoms and five signs (Rudolf et al., 2013). However, in this study, we only used five symptoms and four signs because the data of mid upper arm circumference (MUAC) was not available in hospital. The correlation between the degree of severity and co-morbid of pulmonary MDR-TB was analyzed by Spearman test using SPSS ver. 21.

Results

In this study, from 24 positive MDR-TB samples, the percentage of male patients were 62.5% and the percentage of female patients were 37.3%. A total of 7 MDR-TB patients were also found to have comorbidities of diabetes mellitus, 1 patient had diabetes along with cancer, and 1 patient had HIV/AIDS. A total of 24 sputum samples from MDR-TB patients being examined using PCR were found to be positive for the full *esxA* gene (100%), as indicated by the 580 bp DNA band (Figure 1).

The primer sequences used in this study were designed by using Clone Manager 6, version 6.00 (Table 1). The primer used in the current study annealed to position 223 of the *esxB* gene (Rv3874) and 43 of the *espI* gene (Rv3876) to capture the entire length of the *esxA* gene (Rv3875) and the linker gene flanking the *esxA* gene.

Table 1: Characteristics of primer used for PCR

| Name of Primer | Primer sequence | Primer position | Size of fragmen (bp) |
|--------------------------|--------------------------------------|-----------------|----------------------|
| <i>esxA</i> Full Forward | 5'-GCAATATTCGTCAGGCCGGCGTCCAATACT-3' | 223 | 580 bp |
| <i>esxA</i> Full Reverse | 5'-CGCTGCCATATCGTCCGGAGCTTCCAT-3' | 43 | |

All samples with distinct DNA bands after visualization by electrophoresis were then sequenced by 1st BASE. The result of alignment in BioEdit version 7.2.5 showed that all sample sequences had 100% identity with the wild-type *M. tuberculosis* H37Rv (NC_000962.3) sequence (Figure 2).

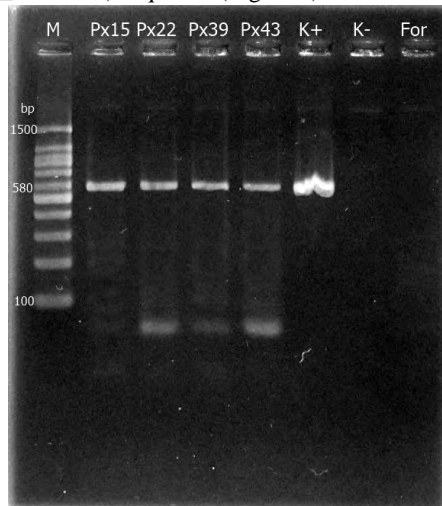


Figure 1: Visualization of PCR results for the *M. tuberculosis* *esxA* gene. A 580 bp DNA band appeared on Px1, Px2, Px3, and Px4. Px: sample amplicon, K+: *M. tuberculosis* H37Rv, K-: reaction mix without DNA template, For: *M. fortuitum*, M: DNA marker Intron Sizer™ -100 bp.

Gene sequencing of samples was also analyzed using the NCBI BLAST program, resulting in all examined samples from the various TB severity classes having 100% identity with *M. tuberculosis* H37Rv. In addition, the identity of samples was also 100% with other species of the *Mycobacterium tuberculosis* complex (MTBC), such as *Mycobacterium caprae*, *Mycobacterium bovis*, and *Mycobacterium africanum*. Three other strains were also found to have identity as high as 99% with the sample sequences that were studied, namely, *M. tuberculosis* strain 96121, *M. tuberculosis* RGTB327, and one other organism from the MTBC, namely, *M. canetti* CIPT 140060008.

The basic data for MDR-TB patients provided various information on symptoms experienced by patients. The data showed that all 24 MDR-TB-positive patients experienced cough, weight loss, dyspnea, fever, and night sweats. Based on the symptoms and signs in patients, the severity degree score based on the Bandim TB scoring method could be determined. The severity based on the Bandim TB scoring method applied in this study was divided into 2 groups: mild grade (SC I) and moderate and severe grade severity groups (SC II and SC III) (Table 2).

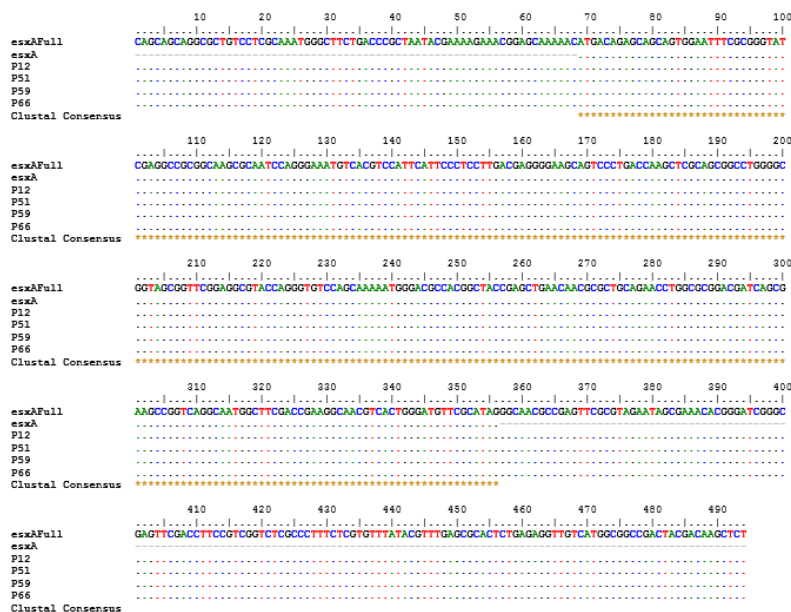


Figure 2: Multiple alignment results using the BioEdit version 7.2.5 program showed 100% identity between samples with wild-type *M. tuberculosis* H37Rv (NC_000962.3). P12, P51, P59, and P66 are samples.

The mild grade group had a score range of 0-5, while the moderate and severe grade group had a score range of 6-11. Bandim TB score data showed that MDR-TB sufferers with mild grade severity had the highest number of patients (15 patients). Data in Figure 2 represent the development of preliminary work (Dewi et al., 2017a). T cell epitope prediction of the *esxA* sequence that acquired in this study has been published before (Dewi et al., 2018).

Table 2: Degree of severity based on Bandim TB score method of MDR-TB patients

| Degree of severity | Number of MDR-TB patients |
|--------------------------------------|---------------------------|
| Mild (SC I) | 15 (62.5%) |
| Moderate (SC II) and severe (SC III) | 9 (37.5%) |
| Total | 24 |

* Bold indicated the highest result

In this study, we also tried to analyze the correlation between severity class and co-morbid of the pulmonary MDR-TB that were collected in this study. The result showed that there is no correlation between them ($p = 0.358$) (Table 3).

Table 3: Correlation between degree of severity and co-morbid of pulmonary MDR-TB patients in Dr Soetomo Hospital, Surabaya, East Java, Indonesia

| Degree of severity | Co-morbid | | | | p value |
|--------------------------------------|-----------|-------------------|------------------------------|----------|---------|
| | None | Diabetes Mellitus | Diabetes Mellitus and Cancer | HIV/AIDS | |
| Mild (SC I) | 8 | 6 | 1 | 0 | 0.358 |
| Moderate (SC II) and severe (SC III) | 7 | 1 | 0 | 1 | |

* Bold indicated the highest result SC, severity class.

Discussion

Demographic conditions such as gender, age, and comorbidity are important data for understanding the transmission and tendency of an illness and disease severity. Patients in this study mostly were male. Worldwide, as many as 5.9 million men and 3.5 million women suspected patients were reported for TB. The WHO stated that 68% of TB cases in Asia occurred in men, while 58% occurred in women. Data in Indonesia from 2015 also showed that total male TB patient population had a greater number (597,000) than females (420,000) (WHO, 2016).

Comorbidity also affected MDR-TB clinical outcomes, and these risk factors could be related to pulmonary tissue immunity. Reports of various studies have shown that many TB cases have comorbidities of diabetes mellitus, HIV, and cancer. Another study also mentioned that HIV and cancer chemotherapy were among the risk factors for *M. tuberculosis* infection (Fisher-Hoch et al., 2008; Parija, 2012; Carroll et al., 2016). This study showed that patients with MDR-TB with comorbid diabetes mellitus had the highest number compared to other comorbidity categories, which was similar to a previous study that stated that the risk of MDR-TB with comorbid diabetes mellitus was 2-3 times higher than that of other cases (Tegegne et al., 2017).

Patients with diabetes mellitus have impaired immunity because glucose levels are not well controlled, thus damaging the function of macrophages in chemotaxis, phagocytosis, reactive oxygen species (ROS) generation, and bactericidal mechanisms (Fisher-Hoch et al., 2008; Tegegne et al., 2017). A study on the effect of diabetes mellitus on tuberculosis treatment outcome in India also showed that TB patients with comorbid diabetes mellitus were known to have a high number of positive sputa after two months of treatment and poor outcomes at treatment completion compared to patients with no comorbid diabetes mellitus (Siddiqui et al., 2016).

Bandim TB scoring could be applied in developing countries and was useful in monitoring patients with TB or MDR-TB currently receiving medication. This method could also play a role in TB screening (Rudolf, 2014). The five symptoms examined consisted of cough, hemoptysis, dyspnea, chest pain, and night sweats followed by six signs: anemia, tachycardia (pulse >90x/min), abnormalities in chest auscultation, fever, body mass index (BMI), and MUAC (Virenfeldt et al., 2014). In this study, measurements of modified Bandim TB with MUAC were not included because MUAC measurement had not yet been applied in Dr. Soetomo Academic Hospital. The highest TB scores in this study came from 8 patients classified as severity class III (SC III), or in the case of this study, they were classified into the moderate and severe group. Present study also showed that there is no correlation between severity class by Bandim TB and co-morbid of patients, which could mean that other factors such as nutritional status is the one which correlate with severity class.

Table 2 shows that 15 MDR-TB patients were categorized as mild, while the other 9 were categorized as moderate and severe. This result indicated that MDR-TB sufferers in this study mostly belong to the mild group, which is in contrast with some studies. A study by Hafez et al. found that the percentage of patients with MDR- and extensively drug-resistant tuberculosis (XDR-TB) categorized as moderate and severe via observing lesion levels on X-ray chest was 44% and 56%, respectively (Hafez et al., 2013).

This phenomenon may be connected to the *M. tuberculosis* strain that infected the patients. A study of mixed infection with Beijing and non-Beijing strains reported that many patients with mixed infection predominantly had the Beijing strain, and its presence was associated with a low bacterial load of the non-Beijing strain in patients, indicating that the Beijing strain may have survival capability and may affect the severity level of patients (Wang et al., 2011). Thus, it was necessary to conduct further research to determine the *M. tuberculosis* strain infecting MDR-TB patients in Dr. Soetomo Academic Hospital and its association with the degree of disease severity.

Additionally, cavitory lesions were often found in cases with MDR-TB; thus, in accordance with a previous study, it was significant to evaluate the degree of MDR-TB infection lung severity using two methods: Bandim TB and chest X-ray reading to obtain more representative results. A specific research design was required to conduct further study in determining the severity class of MDR-TB patients, specifically to determine the correlation between the characteristics of the full *esxA* gene and the severity class of MDR-TB patients.

Further study on the secretion level of proteins affecting virulence and its association with disease severity is essential for understanding the causes of severity differences in MDR-TB patients. Secretion levels of proteins affecting virulence of virulent *M. tuberculosis* strains, such as lipoproteins, system secretions (ESAT-6 and RD-1 members), and *hspX* protein acting as an inhibitor of macrophage antimicrobial effectors, were also assumed to affect the severity of MDR-TB patients. The results from other studies also indicated that ESAT-6 protein had the ability to subvert host immune responses by inhibiting nitric oxide (NO) and ROS generation and inhibiting or triggering apoptotic processes in macrophages, thereby regulating host cells in response to *M. tuberculosis* infection (Xie et al., 2016). In a study using molecular amplification, primers were important for accurately detecting gene targets. Primers used for amplification in this study captured the *esxA* 580 bp gene region. The primers in this study were designed by using sequences from the *esxB* gene (Rv3874) at position 223 for the forward primer and the *espI* gene sequence (Rv3876) at position 43 for the reverse. A previous study only attempted to capture 300 bp, 306 bp, 320, 339 bp, and 351 bp of the *esxA* gene, and the sequence also revealed 100% homology with *M. tuberculosis* H37Rv. However, most of them were used for TB diagnostic development or vaccine development (Gao et al., 2015; Mertaniasih et al., 2016; Soleimanpour et al., 2016; Dewi et al., 2017a). Therefore, the current study attempted to analyze *esxA* gene sequences of longer size, intended to capture the full gene and linker genes flanking the *esxA* gene; thus, the full length of the *esxA* gene could be analyzed.

Several studies have suggested that mutations occurring in bacteria could interfere with its fitness, but in fact, many TB patients developed into MDR-TB patients with varying degrees of disease severity. Previous studies have also stated that the resistant *M. tuberculosis* strains in clinical samples were different from bacteria mutated in the laboratory because the discovery of a resistant *M. tuberculosis* strain in a sample did not necessarily indicate a decrease in growth rate, and the clinical strains still possessed the same transmission ability as antibiotic-sensitive bacteria. In addition, mutations in genes targeted by anti-TB drugs may affect the secretion of virulence factors; thus, it is likely that the *esxA* gene, one of the main virulence genes, is also mutated and affected the virulence of MDR-TB (Melnik et al., 2015; Salvatore et al., 2016).

However, the results of *esxA* gene sequencing showed that the gene in all the samples from both the mild and the moderate and severe groups had 100% homology (Figure 2) with that of *M. tuberculosis* H37Rv (NC_000962.3). The result of alignment using BLAST showed that the sample sequences had 100% homology with the MTBC group, and similarity was not found towards the NTM (nontuberculous mycobacteria) bacterial group. This indicated that no mutation had occurred in the *esxA* gene of MDR-TB from either the moderate and severe group or the mild group. Previous studies analyzing ESAT-6, Ag85B, and Ag85C sequences also showed that in the *esxA* gene, a single-nucleotide polymorphism was not found (Mertaniasih et al., 2016).

Another study analyzing the *esx* gene group in 108 samples showed that there was no variation found in the *esxA* gene. The study also stated that the *esx* genes encoded by the ESX-1 to ESX-4 loci had fewer variations compared to the *esx* genes located outside the loci. The amino acids of ESAT-6 are also known to be conserved among MTBC strains (Uplekar et al., 2011; Solans et al., 2014).

Bold et al. attempted to analyze in vivo growth and in vitro secretion of ESAT-6 and CFP-10 in *M. africanum*, *M. tuberculosis* and a strain of *M. africanum* supplemented with Rv3879 from *M. tuberculosis*. The Rv3879 gene region was a mutated gene in *M. africanum* and was suspected to be the cause of incomplete ESAT-6 secretion. The results showed that the secretion of ESAT-6 and CFP-10 of all observed strains was not different, so it was concluded that the Rv3879 mutation did not affect the ESAT-6 secretion defect (Bold et al., 2012).

This might also be the case in *M. tuberculosis*, in which mutations in genes playing a role in anti-TB drug resistance, such as *rpoB* and *katG*, did not affect the secretion of ESAT-6, thus implying that the *esxA* gene examined in this study had no variation. A study conducted in 2010 also suggested that mutation occurring in the Rv3616c-Rv3617 region of resistant bacteria caused no difference in extracellular secretion of ESAT-6, even though Rv3612c-Rv3616c belongs to the mutated region required for ESAT-6 and CFP-10 secretion (Motiwala et al., 2010).

Another study revealed that ESX-1 secretion was significantly more regulated in *M. tuberculosis* JAL strains (streptomycin-, isoniazid-, and rifampicin-resistant *M. tuberculosis*) than in *M. tuberculosis* H37Rv, *M. tuberculosis* H37Ra, or *M. tuberculosis* strain BND (streptomycin-resistant *M. tuberculosis*). This was evidenced by higher levels of ESAT-6 and CFP-10 protein secreted by the *M. tuberculosis* JAL strain than by the other three strains. ESAT-6 protein secretion also showed no significant difference between *M. tuberculosis* H37Ra and *M. tuberculosis* H37Rv (Jhingan et al., 2016).

Jhingan et al. also added virulence-related proteins, such as Rv2780 (L-alanine dehydrogenase), Rv0126 (TreS), Rv2299 (HtpG), Rv903c (PrrA), Rv3133c (Devr) and Rv0042c, were secreted at low levels in *M. tuberculosis* JAL (Jhingan et al., 2016).

These studies reinforced the assertion that the *esxA* gene sequence was conserved and rarely mutated, even in resistant *M. tuberculosis*. Furthermore, *M. tuberculosis* resistant to more than one anti-TB drug was suspected to have a higher virulence level than *M. tuberculosis* resistant only to one anti-TB drug because it expressed more ESAT-6 protein than did *M. tuberculosis* resistant to one anti-TB drug or *M. tuberculosis* sensitive to anti-TB drugs. In addition, variation among patients from the mild group and the moderate and severe group in MDR-TB samples might be affected by patients' general health or nutrition condition (Chuchottaworn et al., 2015).

A shortcoming of the current study was that no study was made to evaluate the secretion level of ESAT-6 protein, in addition to the incompleteness of the clinical diagnostic component required for Bandim TB analysis, such as MUAC clinical examination data for minimizing confounding factors. Further research on *esxA* protein secretion correlated with the severity of TB disease is required, which might be useful for clinical implementation as a predictor of TB disease severity.

Therefore, further investigation of sequence analysis of other virulence genes, such as Ag85A, Ag85B, Ag85C, *esxB*, *espD*, and other ESX-1 gene clusters, is required in both MDR-TB and TB patients. In addition, according to the findings in this study, the *esxA* nucleotide sequence of samples had 100% identity with the wild type; thus, further research could be conducted to determine the accuracy of the *esxA* gene when used as a TB diagnostic method, which is important especially for developing countries since their population is more likely to be infected by TB.

In addition, it is also crucial to conduct more research related to the role of pathogenesis in the destruction of pulmonary tissue. The application of easy, feasible and routine severity scoring as a standard procedure is important. Severity scoring by examining clinical manifestations has a role in early detection along with diagnosis and is also important for monitoring treatment evaluation.

Conclusion

This study showed that the characteristic nucleotide sequence among the isolates of sputum from MDR-TB patients from all types of disease severity classes revealed no variation or mutation. The full 580 bp *esxA* gene had

100% identity with that of wild-type *M. tuberculosis* H37Rv (NC_000962.3) and other MTBCs. However, no association was found between the nucleotide sequence expression of the full *esxA* gene and the disease severity class using Bandim TB scoring.

Declaration of Conflict of Interest: The authors declare that they have no competing interests.

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