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Cloning of the *pe11* (LipX, Rv1169c) Gene of a *Mycobacterium tuberculosis* Beijing Strain into the pcDNA3.1 Plasmid Vector

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Abstract

Tuberculosis (TB) is an infectious disease caused by *Mycobacterium tuberculosis*. It is a persistent global health problem with a high mortality rate. Currently, TB is controlled by administering the Bacillus Calmette-Guerin (BCG) vaccine, but the effectiveness of its protection varies among individuals in a population. The *pe/ppe* gene family comprises a typical group of genes that play a role in avoiding the host immune response and inducing persistent TB infection. Based on in silico analysis, the *pe11* gene has estimated immunogenicity and potential as a TB seed vaccine candidate. The *pe11* gene from an Indonesian isolate of an *M. tuberculosis* Beijing strain was amplified by polymerase chain reaction (PCR) and inserted into the mammalian expression vector pcDNA3.1. The recombinant vector pcDNA3.1-*pe11* was used to transform Top10 competent *Escherichia coli*. Clones from the transformation were subjected to colony PCR to confirm the direction of the insert. Sequencing was performed to confirm the correctness of the insert sequence. In this study, the *pe11* gene was successfully cloned into the pcDNA3.1 vector in the correct direction to assure PE11 expression. No mutations were found in the *pe11* gene insert, compared with the *M. tuberculosis* H37Rv sequence as the standard. A pcDNA3.1 vector containing the *pe11* gene derived from an *M. tuberculosis* Beijing strain was successfully constructed.

Keywords: Tuberculosis, *M. tuberculosis, pcDNA3.1, pe11*

Introduction

Tuberculosis (TB), caused by *Mycobacterium tuberculosis*, is transmitted through air that has been contaminated with the bacterium. TB infection is a persistent global health problem, especially in developing countries such as India, Indonesia, Nigeria, Pakistan, and South Africa [1]. The World Health Organization reports that there are 10.4 million new TB cases, 1.8 million of them fatal, worldwide, but only 6.1 million cases are detected and officially reported [1–3]. The prevalence of tuberculosis infection places it among the 10 most common causes of death in the world. In Indonesia, it is estimated that one million new TB cases occur each year, and Indonesia was among the five countries with the highest number of TB cases worldwide in 2015–2017 [3–4].

Vaccination is one of the most effective interventions for suppressing TB cases, along with anti-tuberculosis drug treatment [5]. The anti-TB vaccine currently in use is Bacillus Calmette-Guerin (BCG), a live attenuated vaccine derived from *Mycobacterium bovis* [5–7]. However, the BCG vaccine is less able to protect adults and immunocompromised or HIV-positive individuals [5, 7–8]. On the other hand, TB often occurs as a co-infection with HIV [4–5]. The BCG vaccine has varying degrees of efficacy against pulmonary TB infection, especially in developing countries [1, 6] and for populations with TB infection by *M. tuberculosis* Beijing strains [9]. Thus, a better study of new TB vaccine candidates is needed [8, 10].

DNA vaccines are a safer form of the vaccine to give for various groups of people [8, 11–12] and can stimulate the host's humoral and cellular immune responses to various diseases, especially infectious diseases [13–15]. This type of vaccine consists of vectors that are inserted with specific antigen-encoding genes from specific microbes [8, 15]. The vector used must express the inserted gene, which later on can induce an adaptive and cellular immune response, for example, the T-cell response that is needed to make an effective TB vaccine [12, 15]. The vector's selection is adjusted to the target cell for the expression of the antigen-coding gene [8, 15]. The pcDNA3.1 vector is a plasmid commonly used in recombinant protein expression systems in mammalian cells. Moreover, the pcDNA3.1

plasmid is widely used for cancer gene therapy16 and the development of recombinant DNA-based vaccines for infectious diseases.17 In constructing a DNA vaccine, specific antigens are needed that can induce an immune response [8, 15, 18].

The *pe/ppe* gene family is one of the pathogenic features of *Mycobacterium*, comprising 8–10% of the whole genome of *M. tuberculosis.*19 The members and roles of the *pe/ppe* gene family have not yet been fully elucidated, hence the *pe/ppe* family is predicted to contribute to the level of *M. tuberculosis* virulence [20–21]. One gene member of this family, *pe11*, is known to have a major role in directing the occurrence of infection and is considered as one of the factors that allows *M. tuberculosis* to evade the host immune system, especially macrophages [22–24]. In silico analysis shows that PE11 can induce the adaptive host immune response, which leads to its other potential function as a biomarker in extrapulmonary TB in children [25].

Research on the *pe11* gene, especially as a vaccine candidate, is scarce, and no research on *pe11* has been found in Indonesia. The role of PE11 in inducing cytokines such as IL-4, IL-10, IL-1β, and TNF-α has been carried out to observe the role of the *pe11* gene in *Mycobacterium smegmatis* infection in test animals. In this study, PE11 induced IL-4, IL-10, and TNF-α as a cellular immune response [23]. In general, genes in the *pe*/*ppe* family can be developed as candidates for a tuberculosis vaccine; moreover, the protein profile of PE/PPE has the opportunity to be used as a diagnostic tool for *M. tuberculosis* infection26. Based on this fact, there is still an opportunity to conduct research on *pe11* in the framework of protective efficacy against *M. tuberculosis* infection.

This study aims to clone the *pe11* gene from an Indonesian isolate of an *M. tuberculosis* Beijing strain into the pcDNA3.1 vector, which can be used as an expression vector in a mammalian system to produce recombinant PE11 protein. The recombinant plasmid can be subsequently tested in an animal model to analyze its ability to induce immunity and be considered for use as a TB vaccine candidate that can be produced in Indonesia.

Material and Methods

Strains. *M. tuberculosis* Beijing and *Escherichia coli* Top10 strains are stock cultures of the Microbiology Department, Faculty of Medicine, Universitas Indonesia.

DNA Extraction. Genome isolation was carried out using the boiling technique at 95 °C for 30 minutes, followed by centrifugation at 8000 rpm for 10 minutes. The supernatant consisted of genomic DNA, which was removed to a fresh tube and stored at –30 °C until use.

Epitope Analysis. Epitope analysis of *pe11* gene expression was conducted by using the Immune Epitope Database (iedb.org). Analyses were performed by submitting the PE11 amino acid sequences. We performed epitope prediction on T cells, especially on the MHC II binding site, and on B cells. The methods of epitope prediction included a consensus approach that combined NN-align, SMM-align, and combinatorial library methods.

Amplification of the pe11 gene. Amplification of the *pe11* gene was performed using the polymerase chain reaction (PCR) technique with a specific primer that annealed to the target region of the gene. The *Bam*HI restriction site was added to the forward primer. Meanwhile, *the Eco*RI restriction site was added to the reverse region. The sequences of the forward and revers primers were 5′-CGCGGATCCATC.TTTTGTCACCACACGGC-3′ and 5′-CGCGAATTCCT GCCCGCGCG-3′, respectively. The forward primer contained the Kozak sequence. We did not include the complete primer sequences for patent purposes. The PCR reaction consisted of 10 pmol forward and reverse primers, 52 µL nuclease-free water, 25 mM $MgSO_4$, 10 µL dNTP mix, 10 µL DMSO, 10 µL 10X reaction buffer, 2 µL SMO-Hifi DNA polymerase [Smobio], and 10 µL of the *M. tuberculosis* genome to a final reaction volume of 100 µL. The PCR program consisted of an initial denaturation for 2 minutes at 94 °C, followed by 30 cycles of 15 seconds at 94 °C, annealing for 30 seconds at 65 °C, 30 seconds at 68 °C, and a cycle at 68 °C for 1 minute. The PCR product size was confirmed through gel electrophoresis, and the fragment was purified using the GeneJET PCR Purification Kit [Thermo Scientific].

Preparation of the pcDNA3.1 Vector. The pcDNA3.1 [Thermo Scientific] vector is the stock of the Virology and Molecular Laboratory Microbiology Department, Faculty of Medicine, Universitas Indonesia. Plasmids were propagated in *E. coli* strain DH5α on Luria Bertani broth medium containing 100 µg/ml ampicillin and then isolated by using the GeneJET Plasmid Miniprep Kit [Thermo Scientific]. The results of plasmid isolation were observed by 8% agarose gel electrophoresis.

Cloning of *pe11* into the pcDNA3.1 Vector. The PCR product of *pe11* and the pcDNA3.1 plasmid were digested with the restriction enzymes *Bam*HI [Thermo Scientific] and *Eco*RI [BioLabs] in an incubator for two hours at 37 °C, followed by ethanol precipitation with addition of 1 M NaOAC and absolute ethanol. The solution mix was then centrifuged at 13,500 rpm and 4 °C for 15 minutes. The supernatant was discarded, and 70% ethanol was added to the pellet. Centrifugation was performed again for 5 minutes, then the supernatant was removed. The DNA pellet was dried in a DNA dryer for 10 minutes, and finally, 10 µL nuclease-free water was added to the dissolved DNA pellet. The result of this

step was used for ligation. The ligation reaction consisted of pcDNA3.1 plasmid, the *pe11* DNA fragment, 10 µL T4 DNA ligase buffer [Fermentas], 1 µL T4 Ligase enzyme [Thermo Scientific], and nuclease-free water to a total reaction volume of 30 µL. Furthermore, the reaction mix was incubated at 16 °C overnight and used for recombinant plasmid transformation into *E. coli* Top10 competent bacteria.

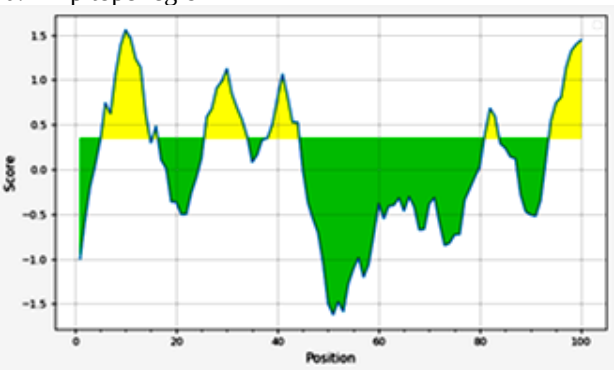
Transformation of Recombinant Plasmid. The recombinant plasmid was used to transform *E. coli* Top10 competent bacteria by using the $CaCl_2$ method, the details of which can be found elsewhere. Bacterial transformants were then spread on LB plate medium containing 100 µg/mL ampicillin, followed by overnight incubation at 37 °C. Colony PCR was performed to obtain bacteria that had been successfully transformed with pCNA3.1-*pe11* recombinant plasmid. Positive colonies were then re-cultured into LB broth medium containing ampicillin 100 µg/ml in a shaking incubator at 37 °C, followed by plasmid isolation. Furthermore, sequencing analysis was conducted to identify the existence of mutations.

Results

Epitope Analysis. The PE11 epitope analysis results showed the regions that could be recognized by the Human Leukocyte Antigen (HLA) or Major Histocompatibility Complex II (MHC II). This complex is on the surface of Antigen Presenting Cells, such as macrophages, B cells, and dendritic cells. The data obtained show that HLA can recognize PE11, including HLA-DR, HLA-DP, and HLA-DQ. The analysis was continued to predict some epitopes for antibody recognition. We used predictive computing using the Bepipred Linear Epitope Prediction method, Sequential B-cell Epitope Predictors, which are sequence-based B-cell epitope predictions using conformational epitopes. The BepiPred-2.0 server predicts B-cell epitopes from a protein sequence, using a Random Forest algorithm trained on epitopes and non-epitope amino acids determined from crystal structures. The results indicate that at least five possible epitopes will be recognized by human B cells (Figure 1). Yellow curves above the threshold line (0.350) indicate the possibility of epitopes recognized by B cells. This suggested the potential of PE11 to induce the host's adaptive immune response.

Amplification of the *pe11* gene. Amplification of the *pe11* gene from the Beijing strain *M. tuberculosis* genome was successfully carried out using the PCR method. As shown in Figure 2, we confirmed the presence of a 324-bp band corresponding to the length of the target *pe11* gene on 1.5% agarose gel electrophoresis. These results indicate that the primers designed and the PCR conditions are suitable to produce the expected PCR products.

a. Epitope region



b. Predicted peptides*

No	Start	End	Peptide	Length
1	6	14	TRPDSIGET	9
2	16	16	A	1
3	26	34	SAHDDGVTP	9
4	39	44	VESPAH	6
5	81	83	QTS	3

**Rewritten from Bepipred Linear Epitope Prediction result*

Figure 1. ***In silico*** **Epitope Prediction using the Immune Epitope Database (iedb.org). a) Prediction of Epitope for HLA, Yellow Area Shows the Region of PE11 that can be recognized as an Epitope for HLA. b) Analysis of Epitope Sequences for B cells.**

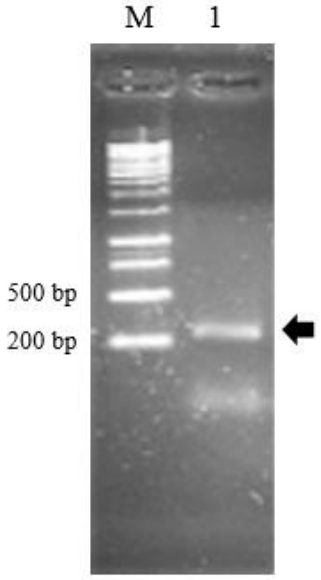


Figure 2. . PCR Product of the *pe11* gene (324 bp) from *M. tuberculosis* Beijing Strain Separated on 1.5% Agarose Gel Electrophoresis. Lane M: 1 kb DNA Ladder; Lane 1: PCR Product of the *pe11* Gene-Coding Region. Black Arrow Showed a Band Correlates to the Length of *pe11*. bp = base pair..

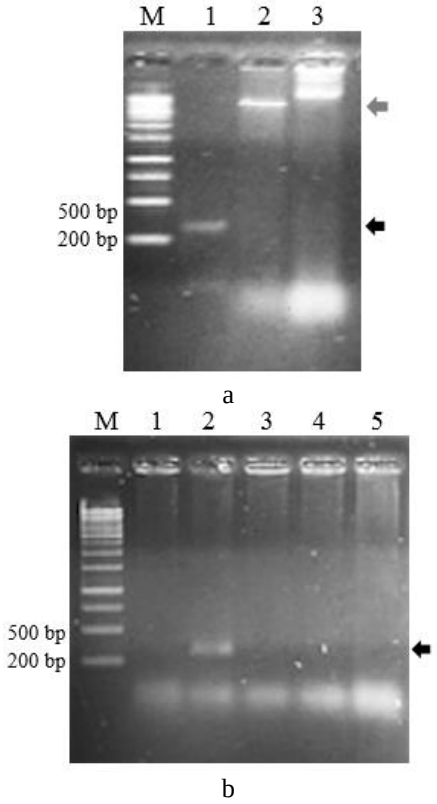


Figure 3. a) *Pe11* gene and pcDNA3.1 Vector Digested with *Bam*HI and *Eco*RI Separated on 1.5% Agarose Gel Electrophoresis. M: 1 kb DNA Ladder. Lane 1: *pe11* gene; and Lane 2: pcDNA3.1 Vector after Digestion with Restriction Enzymes; Lane 3: pcDNA3.1 Undigested. Gray Arrow Shows a Band Correlating to the Length of pcDNA3.1 after Digestion. Black Arrow Shows a Band Correlating to the Length of *pe11*. b) Colony PCR with Primers Specific to *pe11*. M: 1 kb DNA Ladder; Lane 1–5: Colony PCR from *E. coli* Transformation Results. Black Arrow shows a Band Correlating to the Length of *pe11*. bp = Base Pair

Ligation and Transformation of Recombinant Plasmid Into Bacteria. PCR products of *pe11* after isolation and purification of pcDNA3.1 were subjected to digestion using the restriction enzymes *BamH*I and *EcoR*I. We confirmed the digestion result on 1.5% agarose gels, as can be seen in Figure 3a. Although the results of restriction enzyme digestion of *pe11* were not visible, the success of the digestion could be observed in the plasmid pcDNA3.1, where there was a single linear band compared with the uncut plasmid. Digestion by restriction enzymes was followed by ligation of the *pe11* gene into the pCDNA3.1 vector using T4 DNA ligase. After overnight incubation, the ligation product was used to transform *E. coli* Top10 bacteria, which were grown on LB agar containing ampicillin as selective media. Then, colony PCR was performed with specific primers to detect the *pe11* gene insert in the recombinant plasmid. Some colonies produced a DNA band at 324 bp, showing that the *pe11* gene had been successfully inserted into the plasmid vector (Figure 3b). We illustrate the cloning of the *pe11* gene into the plasmid vector pCDNA3.1 in Figure 4. In our construct, the *pe11* gene was inserted downstream of the CMV promoter, with a stop codon added to the downstream region of the *pe11* gene; in theory, only PE11 protein expression would occur. This construct is expected to express PE11 protein in vivo and stimulate an immune response in test animals.

Sequencing analysis of pe11 from pCDNA3.1-*pe11* recombinant plasmid. For the purpose of observing any mutations in *the pe11* gene insert, we isolated two colonies and performed PCR using primers that annealed to upstream regions of the plasmid and reverse regions of *pe11*. The electrophoresis results showed a band of 484 bp correlated to the expected length of the amplicon region (figure not shown), indicating that the direction and location of insertion were correct in the plasmid vector. Then, we sequenced the amplicon of *the pe11* DNA insert and analyzed the thread of sequence. Analysis of the sequencing results showed that no mutations had occurred in the DNA insert, and the sequence was 100% similar to that of *pe11* from *M. Tuberculosis* H37Rv as standard sequences (Figure 5).

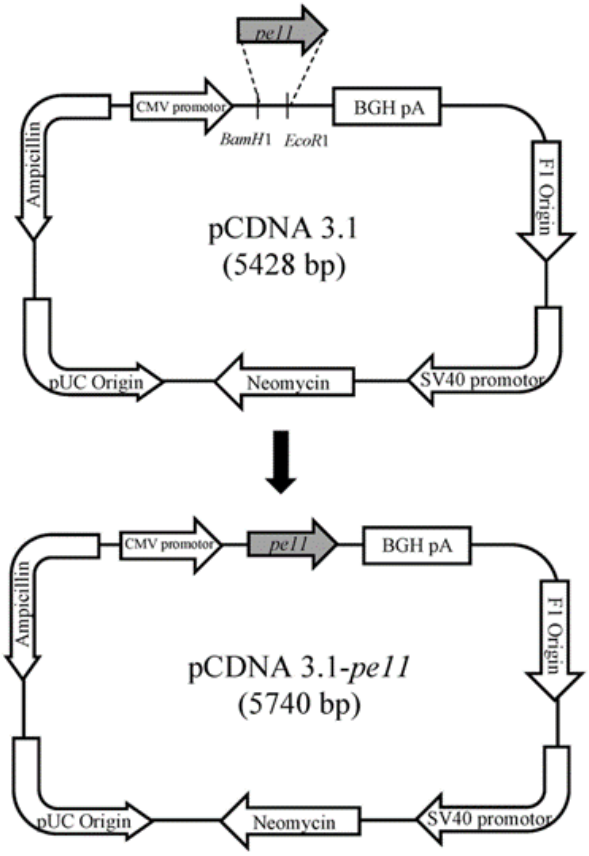


Figure 4. Construction of Recombinant pcDNA3.1 Plasmid Containing *pe11* DNA

```
                ....|....| ....|....| ....|....| ....|....| ....|....|
                       160        170        180        190        200
pe11 H37Rv      -----tgtct tttgtcacca cacggcccga ttcgattggg gaaacggccg
pe11 Beijing    GGCCGTGTCT TTTGTCACCA CACGGCCCGA TTCGATTGGG GAAACGGCCG

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       210        220        230        240        250
pe11 H37Rv      ccaacctcca cgagatcggg gtgacgatga gcgcccatga tgacggggtc
pe11 Beijing    CCAACCTCCA CGAGATCGGG GTGACGATGA GCGCCCATGA TGACGGGGTC

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       260        270        280        290        300
pe11 H37Rv      acgccgctga tcaccaatgt ggaatccccc gcccacgatc ttgtgtccat
pe11 Beijing    ACGCCGCTGA TCACCAATGT GGAATCCCCC GCCCACGATC TTGTGTCCAT

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       310        320        330        340        350
pe11 H37Rv      cgtgacgtcg atgctgtttt ccatgcacgg cgagctgtac aaggcgatcg
pe11 Beijing    CGTGACGTCG ATGCTGTTTT CCATGCACGG CGAGCTGTAC AAGGCGATCG

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       360        370        380        390        400
pe11 H37Rv      cgcgccaggc ccatgtgatc cacgagtcat ttgtccaaac acttcagacc
pe11 Beijing    CGCGCCAGGC CCATGTGATC CACGAGTCAT TTGTCCAAAC ACTTCAGACC

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       410        420        430        440        450
pe11 H37Rv      agcaagactt cgtattggct caccgaatta gccaaccgcg cgggcacctc
pe11 Beijing    AGCAAGACTT CGTATTGGCT CACCGAATTA GCCAACCGCG CGGGCACCTC

                ....|....| ....|....
                       460
pe11 H37Rv      cacctag--- ---------
pe11 Beijing    CACCTAGGAA TTCGCGAGA
```

Figure 5. Sequencing Analysis of the *pe11* Gene from the pcDNA3.1-*pe11* Recombinant Plasmid. *pe11* H37Rv = p*e11* Sequence from *M. tuberculosis* H37Rv from http://genolist.pasteur.fr/TubercuList/; *pe11* Beijing = Sequencing Result of *pe11* from pCDNA3.1-*pe11* Recombinant Plasmid

Discussion

Studies on *pe11* require additional exploration, especially to discuss the potential of *pe11* as a DNA vaccine candidate to prevent TB infection and determine the immunogenic level of *pe11* to trigger the host's adaptive immune response. We attempted to analyze an epitope of the *pe11* protein, which has a molecular weight of 10.8 kDa with a total of 100 amino acids by using the Immune Epitope Database (iedb.org). Analysis using this database is considered quite representative, and *in vitro* results were concordant with *in silico* analysis in our experience with an RpfD protein experiment (unpublished data). In this case, the results of *in silico* analysis indicated that the *pe11* protein has an epitope to induce humoral and cellular immune responses in which there are recognition epitopes for HLA and B cells. As we know, the HLA will present antigens to CD4+ helper T cells, which can then activate T cells, and in a certain mechanism, pathways can also stimulate B cells to produce antibodies that are specific to these antigens [27–28].

To study the potential of *pe11* as a seed vaccine candidate, we constructed a plasmid recombinant of p*e11* into the pCDNA3.1 plasmid. We amplified p*e11* from an Indonesian isolate of an *M. Tuberculosis* Beijing strain using PCR. The selection of Beijing strains from Indonesian isolates was based on several studies stating that most of the *M. tuberculosis* isolates in Indonesia and some countries in Southeast Asia are of the Beijing strain [29–30]. They also refer to the induction of host immune responses, clinical features, epidemiology, and pathogenic characteristics that determine the manifestations of infection that occur.

Various strains of *M. tuberculosis* determine this [9, 29, 31–32]. Furthermore, BCG vaccine administration in populations with TB infection by Beijing strains is not effective for prevention of TB infection by the strains other than Beijing [9]. Thus, the selection of Beijing strains in this study is expected to produce a TB vaccine candidate more specific for the pathogenic character of *M. tuberculosis* in Indonesia and be an incentive for independent production in Indonesia.

The selection of a vector in this study was adjusted to future goals, specifically applying recombinant vectors with expression systems in mammalian cells to be used and tested as TB vaccine seeds [13,16,18,33]. The pcDNA3.1 vector has a cytomegalovirus (CMV) promoter. The CMV promoter is one of the strong expression promoters derived from human cytomegalovirus. This promoter can drive the expression of exogenous genes in mammalian cells; therefore, transfection with this vector is the preferred method for expressing target genes in mammalian cells [33–34]. In the region downstream of the CMV promoter where the *pe11* gene was inserted. Moreover, pcDNA3.1 has a bovine growth hormone region, which signals polyadenylation and is a transcription termination sequence to increase mRNA stability [33, 35]. The presence of pUC origin is also useful for selection and for facilitating the replication of *E. coli* bacterial cells [33]. Some members of our previous research team have proven the success of cloning various genes into pcDNA3.1, including *rpfB* and *rpfD* genes, which shows that this vector can be a good choice for cloning various genes of *M. tuberculosis* [36–37].

As mentioned above, the selection of *M. tuberculosis* Beijing strains from Indonesia is expected to provide unique characteristics of the recombinant protein produced. However, it was beyond our expectations that there was no difference found to the standard sequence of *M. tuberculosis* H37Rv. It might be that the resulting protein PE11 would have the same structure to that of other TB strains. This could potentially lead to the same immune response among *M. tuberculosis* strain infection. Moreover, we think this is an advantage that the *pe11* will protect against any infecting strains of *M. tuberculosis*. The vaccine produced subsequently can be applied in a variety of different *M. Tuberculosis* infection settings.

Conclusion

We succeeded in constructing the pcDNA3.1 vector encoding *pe11* derived from an *M. tuberculosis* Beijing strain. However, further research is needed to examine the expression of *pe11* and the response of adaptive immunity in an animal testing model. This study is a promising first step toward a TB vaccine candidate produced in Indonesia.

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