

4-1-2018

Development of a Tuberculosis Vaccine Seed: Construction of Resuscitation-Promoting Factor B DNA Vaccine and its Expression in Vitro and in Vivo

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Recommended Citation

Saraswati RD, Rukmana A, Fithriyah F, Rakhmawati A. Development of a Tuberculosis Vaccine Seed: Construction of Resuscitation-Promoting Factor B DNA Vaccine and its Expression in Vitro and in Vivo. Makara J Health Res. 2018;22.

Development of a Tuberculosis Vaccine Seed: Construction of Resuscitation-Promoting Factor B DNA Vaccine and its Expression in Vitro and in Vivo

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Abstract

Background: Tuberculosis (TB) is a chronic infection disease caused by *Mycobacterium tuberculosis* (*Mtb*) and has a high death-rate worldwide. Bacillus Calmette-Guerin is the only TB vaccine which is currently available with several drawbacks, such as its different efficacy for different individuals, lack of protection for lung TB in adults and subsequent reactivation which lead the research for novel TB vaccine approach. Resuscitation-promoting factor (rpf) protein in *Mtb* is a protein cluster which play a big role in TB dormancy during latent infection. Member from this cluster protein is rpfB which shows the greatest biological and immunological characteristics among other proteins in the rpf family, now is widely explored as novel TB vaccine candidate. **Methods:** In this study, the *rpfB* gene of the *Mtb* Beijing strain was amplified using PCR and then cloned into pcDNA3.1 plasmids. The ability of recombinant pcDNA-rpfB to induce humoral immune response was tested through Balb/C mice immunization. **Results:** A positive recombinant rpfB protein ~66 kDa was detected through western blot analysis using immunized mice sera. Meanwhile, recombinant pcDNA-rpfB was transfected in to CHO-K1 mammalian cell line and recombinant rpfB antigen expression was confirmed through immunostaining. **Conclusions:** Therefore, we have successfully express the recombinant rpfB proten of M.tb strain Beijing in mammalian expression system which proven to be antigenically induced humoral immune response in mice model.

Keywords: CHO-K1, DNA vaccine, *Mycobacterium tuberculosis*, *rpfB* gene

Introduction

Tuberculosis (TB) is a chronic infectious disease caused by *Mycobacterium tuberculosis*. According to the 2015 World Health Organisation (WHO) data, TB is one of the top 10 infectious diseases worldwide, with a death-rate of 1.8 million people yearly, higher than that of HIV and malaria. Countries with the greatest number of TB patients include India, Indonesia, China, Nigeria, Pakistan and South Africa.¹ Most individuals infected with *M. tuberculosis* experience latent TB infection, meaning they do not show symptoms and do not transmit the disease. In latent TB patients, bacteria are dormant and do not cause clinical symptoms but can be reactivated.²

TB can be prevented by vaccinating those who have not been infected by the *M. tuberculosis* bacteria. The Bacillus Calmette–Guerin (BCG) is the only anti-TB vaccine used worldwide nowadays. This vaccine originated from an attenuated *Mycobacterium bovis* strain³. BCG is a very secure vaccine, relatively cost-efficient and stable at room temperature,⁴ however, its protection capability varies, ranging from 77% in

England to 0% in Chinglepur, India and some places in the USA.^{5,6}

The BCG vaccine is useful to protect from severe childhood TB, but the immune effect it produces only lasts for 10 to 15 years and does not provide protection against lung disease in adults.⁴ In addition, the BCG also does not provide protection against TB latent infection and its subsequent reactivation.⁵ This has prompted new research to develop anti-TB vaccines that are more effective.

Preliminary studies and bioinformatic analyses have identified some *M. tuberculosis* genes as possible new vaccine candidates. Possible vaccine candidate antigens may originate from the resuscitation-promoting factor (*rpf*) gene family. The *rpf* genes expresses rpf proteins that play a role in bacteria recovery (resuscitation) in the latent to replication phases.⁷ *M. tuberculosis* expresses five different Rpf proteins, namely RpfA–E, all of which have resuscitation activity and are immunogenic in mice.⁷ RpfB protein, one of the five Rpf proteins produced by *M. tuberculosis*, plays an important role in dormant bacteria recovery and growth. Analysis of

RpfB protein composition has shown that this protein is a membrane protein with B- and T-cell multiple epitopes.^{8,9} Previous research showed that the RpfB protein has the highest biological and immunological characteristics among other the Rpf family proteins.¹⁰ Deletion of the *M. tuberculosis rpfB* gene causes a delay in reactivation in mice infected with *M. tuberculosis*. Furthermore, mutations in this gene can reduce the growth levels of the *M. tuberculosis* H37Rv strain. This makes RpfB a suitable antigen for the development of a new or as complementary TB vaccine.¹⁰

Meanwhile, the newest method in vaccine development uses DNA as a vaccination platform, known as a DNA vaccine. DNA vaccines consist of a bacterial plasmid with a strong eukaryotic promoter gene (such as an enhancer element from cytomegalovirus), a gene that encodes an immunogenic protein, a polyadenylation signal and a transcriptional termination sequence.¹¹ Although there have been many studies of the RpfB protein, to our knowledge, there has been no research into the development of a TB vaccine based on DNA using the RpfB protein especially in Indonesia. Therefore, this study aimed to construct a recombinant DNA-*rpfB* vaccine originating from the Beijing strain of *M. tuberculosis*, local to Indonesia, and analyse its expression capability in vitro, as well as the humoral immune response generated, in order to provide additional data to develop a new TB vaccine seed.

Methods

***rpfB* gene amplification.** Forward and reverse primers were independently designed by using BioEdit version 3.0 software. The primer pair was designed specifically to the open reading frame of *rpfB* of the *M. tuberculosis* Beijing strain. In the forward primer, a *Hind*III restriction site was inserted, and in the reverse primer, *Eco*R1 was inserted. The sequences were: *rpfB*-F, 5'-CGCAAGCTTATCATGGCG...TTG.....-3' and *rpfB*-R, 5'-CGCGAATTC...GCG.....-3'. The unfilled dots show base components that are not provided for patent purposes.

Cloning process of *rpfB* gene with pcDNA3.1. The *rpfB* gene and pcDNA3.1 plasmid were cut using the *Eco*R1 (Fermentas) and *Hind*III (Fermentas) enzymes and then ligated using T4 DNA ligase (Fermentas). The ligation products were then transformed into the *Escherichia coli* DH5a strain. The success of this recombinant plasmid transformation was confirmed by PCR analysis of a bacterial colony that probably contains *rpfB* gene insert in plasmid, plasmid cutting with *Stu*I restriction enzyme and sequencing. The positive colonies were then grown in 10 mL LB medium with 100 µg/mL ampicillin overnight at 37 °C, and then 1 mL of culture dissolved in LB medium contain glycerol 10% for isolate stock.

Mouse immunisation. Preparation of plasmid for immunization were done by growing *E. coli* DH5a strain consist of recombinant plasmid or pcDNA3.1 itself in 100 mL LB medium with 100 µg/mL ampicillin at 37 °C overnight. The bacterial cells were harvested and then plasmid were isolated using a GeneJET Plasmid Midiprep Kit (Thermoscientific). Concentration of plasmid were adjust to 100 µg in 100 µL of TE buffer. Immunisation was performed on 6 to 8-week-old male Balb/C mice. The mice were divided into three treatment groups containing six mice each. Mice were injected with pcDNA3.1 plasmid only, Tris-EDTA (TE) or the pcDNA3.1-*rpfB* recombinant plasmid. Plasmids were injected intramuscularly using a disposable syringe with a 27 G needle on either the right or the left thigh muscle. A booster was performed on days 7, 14 and 21 and blood was also drawn from the mice using the retro-orbital technique. Mice were anaesthetised via ether inhalation before blood samples were taken. The blood sample volume was 130 µL, around 10% of the total blood volume. Fifteen days after the last immunisation, blood was redrawn using the retro-orbital technique. Blood collection after immunisation was performed to obtain serum that was expected to contain antibodies, which were then used in expression testing using immunostaining and Western blot. All mice experiments were approved through Ethical Approval No. 984/UN2.F1/ETIK/2016 from Ethical Committee Faculty of Medicine, Universitas Indonesia.

Humoral immune response test in mouse serum (Western blot). The RpfB protein was obtained from the pGEX-*rpfB* BL21 strain of *E. coli* (constructed by Rukmana et. al., 2014, Submitted) and its expression was induced by the addition of 0.1 mM isopropyl-β-D-galactosidase (IPTG). The protein profile was then analysed and visualized using 15% SDS-PAGE followed by staining.

The mouse serum was analysed using Western blotting to SDS PAGE where GST-RpfB, and GST protein were run. Briefly, The protein samples were transferred from the SDS gel using the semi-dry transfer method with a bottom-to-top sequence of Whatman paper, Hybond-N-Extra membrane, polyacrylamide SDS gel and Whatman paper. The transfer process was performed using Trans Blot SD-Semi Dry Electrophoresis Transfer (Biorad) in a transfer buffer (2.5 g glycine, 5.8 g Tris-Base, 200 mL methanol and H₂O to a final volume of 1 L), 5 V voltage and 0.1 A for 2 h. After the protein was successfully transferred into the membrane, the membrane was then soaked in blocking solution that contained 5% skimmed milk in 1× Tris-buffered saline and Tween 20 (TBST) solution and incubated overnight at 16 °C. The solution was then removed and changed to 1× TBST solution containing 1% skimmed milk and mouse serum diluted to 1/1000. As a control, the peroxidase/GST-labelled anti-GST antibody (AbCam) was used diluted to 1/5000

and incubated for 1 h with slow shaking at 2 rpm. Control membranes (anti-GST) were then washed three times with 1× TBST (5 min each wash) at 2 rpm at room temperature. On the other hand, the membrane with mouse serum was resoaked in 1× TBST that contained 1% skimmed milk with immunoglobulin G (IgG) horseradish peroxidase (HRP) mouse α -goat antibody (Invitrogen) diluted to 1/5000, incubated for 1 h at room temperature and slowly shaken at 2 rpm. After that, the membrane was washed three times with 1× TBST (5 min each wash). Finally, both membranes were soaked in TMB substrate (KPL) and incubated until the protein band was visible. The membrane was photographed for documentation purposes.

pcDNA3.1-*rpfB* expression analysis in mammalian cells. The pcDNA3.1-*rpfB* recombinant plasmid transfection into Chinese hamster ovary (CHO-K1) cells was performed using the cationic lipid method using lipofectamine LTX and Plus Reagent (Invitrogen). The cells were grown in 24-well plates for 24 h in an incubator with 5% CO₂ CHO-K1 at 37 °C in MEM (Sigma) supplemented with 10% foetal bovine serum (FBS). Cell growth inside the plate was observed until a minimum of 80% confluence was reached. This cell concentration is optimum condition for transfection process.

The transfection was performed in 1.5-mL eppendorf tubes with 200 μ L of OPTIMEM medium (Gibco). First, 500 ng of the recombinant plasmid was added, then 0.3 μ L of Plus Reagent was added into the mixture and incubated for 5 min at room temperature. Then, 1 μ L of lipofectamine was added and incubated at room temperature for 30 min. The medium of the CHO-K1 cells to be transfected was removed and the wells were washed three times with OPTIMEM medium. The plasmid mixture was added to the CHO-K1 cells and incubated with 5% CO₂ at 37 °C for 4 h. Afterwards, the lipofectamine solution was removed and 200 μ L of MEM supplemented with 10% of FBS was added into the wells and incubated overnight in a CO₂ incubator at 37 °C.

After 24 hours, the cells were washed three times with 1× phosphate-buffered serum (PBS) and then dried overnight at room temperature. The cells were then fixed by adding 500 μ L of cold absolute ethanol and incubating at -20 °C for 30 mins. The ethanol was then removed and the cells were dried at room temperature. The fixed cells were stained by adding serum diluted to 1/100 from immunised mice into 200 μ L of 1× PBS and incubating for 1 hour at room temperature. This was followed by three washes using 1× PBS (5 mins each wash). Then, 200 μ L of secondary antibody (IgG HRP mouse α -goat diluted to 1/500 with 1× PBS) was added into the cells and reincubated for 1 hour at room temperature. After that, the cells were washed three

times with 1× PBS (5 min each wash), added 200 μ L of Nacalai substrate and incubated for 30 mins. The substrate solution was removed and the cells were washed with 1× PBS and observed under a microscope using a 40× magnification.

Results

Recombinant pcDNA3.1-*rpfB* construction. *rpfB* gene was successfully amplified in size of 1101 bp using specific primers with *EcoR*I and *Hind*III restriction enzyme (data not shown), thus it was cloned in to pcDNA3.1 expression plasmid through heat shock method in to competent *E. coli* DH5 α bacteria. Following transformation, a total 95 colonies were obtained, and 42 colonies were chosen randomly for PCR screening using insert and plasmid primers. Four recombinant plasmid from positive colonies of PCR screening (data not shown) were further analysed using *Stu*I restriction enzyme. This enzyme has only one restriction site in pcDNA3.1 plasmid, thus recombinant plasmid with insert will show higher band (6517 bp) than the original plasmid (5428 bp) as the result were depicted in Figure 1. Furthermore, 2 out of 4 recombinant plasmids were chosen for sequencing analysis as the result showed that there were no mutations or insertions in the whole recombinant DNA construction (sequence data was not shown due to patent issue).

Humoral immune response test in mouse serum (Western blot). To assess the ability of pcDNA3.1-*rpfB* DNA vaccine to induce humoral immune response, western blot analysis was performed using pGEX-*rpfB* expression system and mice immunization sera. As depicted in Figure 2, native pGEX6p-1 plasmid is not producing any band in both induced and non-induced IPTG (lane 1 and 2). Meanwhile, recombinant pGEX-*rpfB* plasmid was shown to give positive single protein band between 50 – 70 kDa, predicted as RpfB-GST recombinant protein (66 kDa) in both induced and non-induced IPTG (lane 3 and 4).

This result indicated that pcDNA3.1-*rpfB* DNA vaccine is able to induce humoral immune response in mice which recognize specifically in to RpfB protein since pcDNA3.1-*rpfB* DNA vaccine was constructed without any additional tag protein correspond to the absence of any band in native pGEX6p-1 lanes. The presence of positive band was shown in pGEX-*rpfB* non-IPTG induced (lane 3) may be the result of any basal expression system that happen inside the cells which allow the RpfB-GST protein expression. Thus, this expression was highly determined by the presence of IPTG inducer in the system as thicker target band protein was shown (lane 4). This western blot analysis was also done using anti-GST monoclonal antibodies (Abcam) as control.

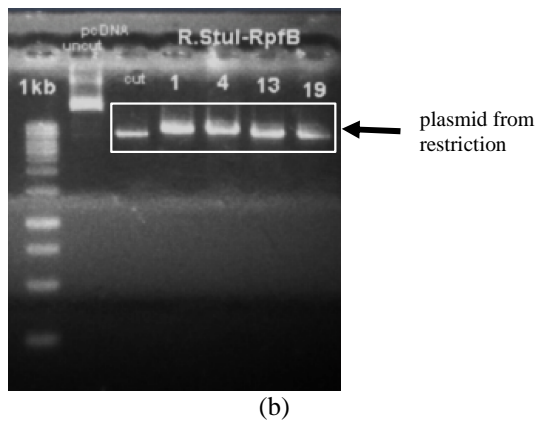
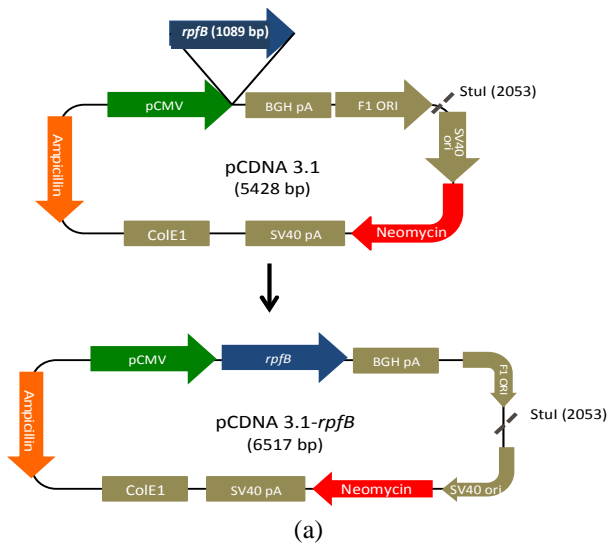


Figure 1. a). Construction of Recombinant DNA Plasmid. b). Electrophoresis of *StuI* Restriction Result (black arrow). M, Marker for 1 kb; Lane 1, pCDNA3.1 Plasmid; Lane 2, pCDNA3.1 Plasmid Cut by *StuI* Restriction Enzyme; Lanes 3–6, pCDNA3. 1-*rpfB* Recombinant Plasmid cut by *StuI* Restriction Enzyme

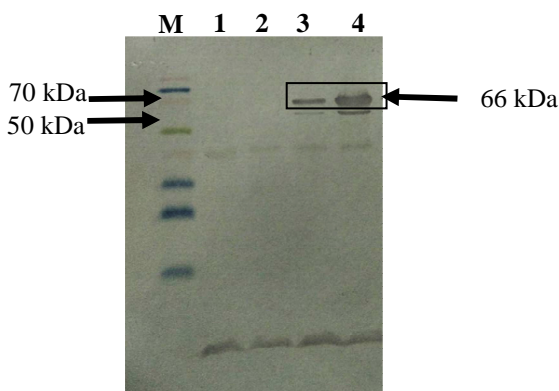


Figure 2. Western Blot Result of RpfB Protein. M, Protein Marker; Lane 1, GST without IPTG Induction; Lane 2, GST with IPTG Induction; Lane 3, RpfB-GST without IPTG Induction; Lane 4, RpfB-GST with IPTG Induction

GST is a protein from *Schistosoma japonicum*. It is a natural protein with a molecular weight of 26 kDa and consists of 220 amino acids. In general, GST is used to produce a fusion protein, which is attached to the protein at the end of the N-terminus.¹² Western blot analysis using anti-GST monoclonal antibodies resulting similar band size (range at 50–70 kDa) on lane 3 and 4 and single band at range of 25–30 kDa on lane 2, indicating the expression of protein fusion of RpfB-GST (data not shown).

pcDNA3.1-*rpfB* expression analysis in mammalian cells. The pcDNA3.1-*rpfB* recombinant plasmid expression was confirmed in a mammalian expression system using CHO-K1 cells and then analysed by immunostaining the transfected cells. Immunostaining was conducted using mouse serum that had been immunised with pcDNA3.1-*rpfB* and confirmed to contain anti-RpfB antibodies. The expression result analysis is shown in Figure 3. Figure 3A is a negative transfection control, where CHO-K1 cells were transfected using native pcDNA3.1 plasmid and stained with the immunized mice sera resulting no violet stained cells observed under the microscope. In figure 3B, is a positive control of transfected CHO-K1 cells with pcD2ME (recombinant pcDNA3.1 plasmid containing prM/E dengue virus gene expression system)¹³ and stained with dengue patients sera resulting violet stained cells. On the other hand, pcDNA3.1-*rpfB* was transfected in to CHO-K1 cells and stained using immunized mice sera which also resulting violet stained cells (Figure 3C). Violet stained cells which observed is the result of chemical reaction between conjugated secondary horseradish peroxidase enzyme and diaminobenzidine substrate, indicating the recombinant rpfB antigen was expressed inside the cells.

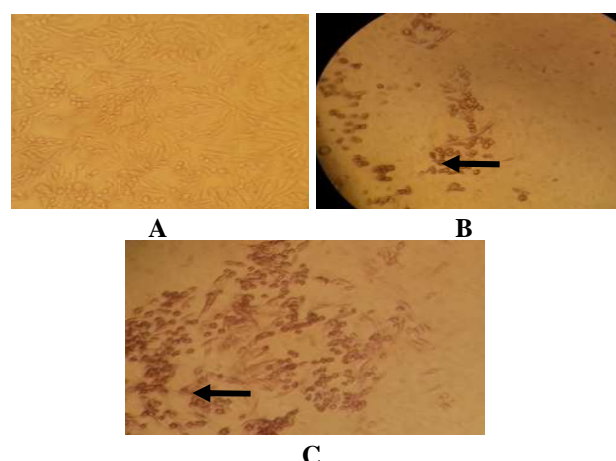


Figure 3. CHO-K1 Cells Transfected with Recombinant Plasmid. A, Negative Control (CHO-K1 Cells Transfected with the Empty pcDNA3.1 Plasmid); B, Positive Control (CHO-K1 Cells Transfected with Dengue prM-E Recombinant Plasmid); C, CHO-K1 Cells Transfected with the pcDNA3.1-*rpfB* Recombinant Plasmid

Discussion

TB is an infectious disease caused by *M. tuberculosis* bacteria. According to the WHO, until now, TB is one of the most infectious diseases and has a high mortality rate worldwide.¹ One approach to prevent TB is vaccination or healthy lifestyle, as well as sanitation improvement in public areas. The BCG vaccine is currently the only TB vaccine used, although its effectiveness is diminishing over time and its efficacy differs in some areas. In addition to its efficacy problem, the BCG vaccine does not provide protection against latent TB infection and subsequent infection reactivation.^{3,4}

Research into possible new TB vaccine candidates has been conducted around the world, especially where the host immune response to TB is known. Most of *M. tuberculosis* infection is manifested as latent infection, where bacteria is in dormancy and regulates survival genes within its genome to adapt the host internal environment and to evade host immune system. Resuscitation-promoting factor (Rpf) is one gene cluster which is well known to play a role in *M.tb* dormancy. The proteins encoded by the *rpf* genes are important to the survival of the bacteria in the latent condition.^{5,6} According to previous research, the RpfB protein has the highest biological and immunological characteristics among other proteins of the Rpf family.⁷ This makes the *rpfB* protein is an appropriate target to be developed as an antigen in designing a TB vaccine so that it can elicit an immune response to the latent *Mtb* infection, enabling dormant bacteria to be eliminated and subsequent TB reactivation to be avoided.⁸

DNA-based vaccines are currently under study. The advantages of this vaccine approach is only containing a foreign DNA substance which relatively safe, can induce both humoral and cellular immune response, easy to design and to be handled for their stability at room temperature.⁹ This study aimed to target a gene from the *rpf* family of the *M. tuberculosis* Beijing strain isolated in Indonesia to produce an *rpfB* single DNA vaccine. This was tested in male Balb/C mice to observe the humoral immunity response as well as the *rpfB* gene expression in a mammalian expression system by transfection.

The *M. tuberculosis* Beijing strain used in this study was obtained from the Microbiology Department culture collection of Medical Faculty, Universitas Indonesia. *M. tuberculosis* Beijing strain itself is the most dominant TB strain in Asia, especially Indonesia, and has characteristics such as it is a more virulent strain and resistant to multiple anti-TB medicines. This local isolate utilisation is one approach to support the government policy about independently supplying raw drug materials and vaccines, as stated in the Strategic Plan Development of pharmaceutical industry and health equipment of the Ministry of Health, 2016.

Therefore, the seed vaccine resulting from this study may eliminate the dependence on imported materials.¹⁴

In this study we have developed a novel TB DNA vaccine, pcDNA-*rpfB*, which had been proven to be expressed in mammalian cell line and able to induce humoral antibody response in mice. Similar results were obtained by Lee et al. (2014), who also used the *rpfB* DNA vaccine approach obtained from *Mtb* H37Rv strain.¹⁵ The DNA vaccine used was pcDNA3.1-*rpfB* was also injected to mice, meanwhile the antibody response was tested by enzyme-linked immunosorbent assay. From previous study, specific response against RpfB protein was formed as IgG antibody classes.¹⁶

The anti-RpfB antibody was formed as a humoral immune response generated by mice as a result of the *rpfB* DNA vaccine. Theoretically, after the DNA vaccine was injected into the tissue, the DNA plasmid would replicate autonomously to produce foreign protein that was antigenic. The foreign protein then induced B cells to produce antibodies for the antigen. The DNA vaccine not only stimulated the humoral immune response through antibody formation, but also stimulated a cellular immune response through T-cell activation. This provided an advantage to the infections caused by intracellular pathogen because cytotoxic T cells could kill infected cells or activate cytokine production and, therefore, stop the infection spreading.¹⁷

Mammalian cells have sophisticated expression systems, such as chaperones, attachment sites, apparatus for protein secretion and post-translational modifications, to help proteins to fold precisely.¹⁵ Previous research has demonstrated that the RpfB protein of *M. tuberculosis* was successfully expressed in prokaryotic system such as *E. coli*, and isolated and purified.¹⁸ As a prokaryotic expression system, *E. coli* cannot perform glycosylation, which is important for post-translation modification, especially for protein that exists inside the cell membrane, as RpfB protein is positioned inside the cell^{19,20} as well as being secreted out of cells.¹⁵ Therefore, by using mammalian expression system, we hope that our recombinant protein product can be folded and modified correctly, better than prokaryotic expression system in order to obtain better protein antigenicity and immunogenicity.

Conclusions

We concluded that the *rpfB* gene from the Beijing strain of *M. tuberculosis* can induce a humoral immune response in mice injected with the pcDNA3.1-*rpfB* recombinant plasmid and that the protein was successfully expressed in the mammalian cells. We hope that these preliminary data can be used in future research as an approach to develop a TB vaccine seed using the Beijing strain from the local isolate.

Acknowledgement

None.

Funding

This study is funded by Kemenristekdikti through Hibah Kompetensi 2015-2016 fiscal year.

Conflict of Interest Statement

None to declare.

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