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Abstract

Tuberculosis (TB) is an infectious disease caused by *Mycobacterium tuberculosis* (*M. tuberculosis*). TB is still a major health problem. The Bacillus Calmette-Guérin (BCG) vaccine is the only one available for TB and is known to confer variable levels of protection. Because of this variability, a new vaccine is needed to control TB. Proteins secreted by *M. tuberculosis* are known to induce protective immunity. Within the genome of *M. tuberculosis,* there is a family of proteins called resuscitation promoting factor (Rpf), which plays a role in the reactivation of *M. tuberculosis*. RpfD is a member of the Rpf family that has been shown to be immunogenic, making it suitable for use as a TB vaccine. The *rpfD* gene of the *M. tuberculosis* Beijing strain from the bacterial stock of the Department of Microbiology at the Medical Faculty of the Universitas Indonesia was amplified using polymerase chain reaction (PCR) and then inserted into the mammalian expression vector pcDNA3.1(+). Then, the pcDNA3.1(+)-*rpfD* vector was transformed to *Escherichia coli* DH5α. A 465-bp target fragment was obtained, and the accuracy of the cloning was confirmed using colony PCR, restriction enzyme digestion, and sequencing. We expect that this recombinant plasmid will induce immunity in future animal models and thus will prove itself to be a candidate for an *M. tuberculosis* vaccine.

Abstrak

Konstruksi vektor pcDNA 3.2 pembawa gen penyandi RpfD dari Mycobacterium tuberculosis. Tuberkulosis (TB) adalah penyakit infeksi yang disebabkan oleh *Mycobacterium tuberculosis*. TB masih menjadi permasalahan kesehatan yang besar. Vaksin BCG (Bacillus Calmette-Guerin) adalah satu-satunya vaksin yang tersedia untuk TB yang telah dipakai dan memiliki daya proteksi yang berbeda-beda diantara negara pengguna. Protein yang disekresikan oleh *M. tuberculosis* diketahui memiliki kemampuan untuk menstimulir sistem imunitas tubuh. Salah satu keluarga protein *M. tuberculosis* yang diberi nama *Resuscitation promoting factor* (Rpf) memainkan peranan dalam re-aktivasi bakteri ini dari kondisi latent. *Resuscitation promoting factor* D (RpfD) menunjukkan sifat imunogenik sehingga memiliki potensi sebagai vaksin TB. Gen *rpfD* dari *M. tuberculosis* strain Beijing yang merupakan stock Departemen Mikrobiologi Fakultas Kedokteran Universitas Indonesia diperbanyak menggunakan *polymerase chain reaction* (PCR) kemudian disambungkan kedalam vector pcDNA3.1(+) yang dapat melakukan ekspresi protein pada sel mamalia. Selanjutnya pcDNA3.1(+)-*rpfD* ditransformasikan kedalam *Escherichia coli* strain DH5α. Fragmen DNA sepanjang 465 bp berhasil didapatkan pada klon hasil transformasi, fragmen tersebut kemudian dikonfirmasi dengan sekuensing untuk mengetahui ada tidaknya mutasi dan pemotongan dengan enzim restriksi untuk mengetahui kebenaran arah insersi gen *rpfD* pada klon. Kami berharap plasmid rekombinan yang didapatkan pada studi ini dapat menstimulasi sistem imunitas pada model hewan uji sehingga menjadikannya sebagai kandidat vaksin tuberkulosis.

Keywords: Tuberculosis, M. tuberculosis, Mammalian expression vector pcDNA3.1(+), Resuscitation promoting factor D (rpfD)

Introduction

Tuberculosis (TB) is an infectious disease caused by *Mycobacterium tuberculosis* (*M. tuberculosis*). TB has existed for millennia and remains a major global health problem, affecting millions of people every year. TB is one of the top nine causes of death for a single infectious disease. In 2016, TB caused 1.3 million deaths, not including an additional 0.4 million deaths among HIV-positive people [1].

More than two-thirds of the global TB burden has been reported as being in Africa and Asia, with India, Indonesia, and China being the three countries that have the highest number of TB cases in the world [2]. In Indonesia, about 1,017,378 new active TB cases were noted in 2015, including multidrug-resistant TB. Because of the number of people infected, TB is a burden on the national economy [3].

TB infection can be prevented by vaccination. The current TB vaccine is an attenuated live vaccine derived from *Mycobacterium bovis* Bacillus Calmette-Guerin (BCG). BCG is known to provide protection against meningeal and miliary TB in infants and children. However, it provides inefficient and inconsistent protection against adult pulmonary TB. Over the years, numerous hypotheses have been proposed to explain the variability in the effectiveness of BCG protection, which can vary from 0–80% [4-7]. In addition, BCG can be virulent and can cause disease in immuno-compromised individuals, for example, those with AIDS [8]. Therefore, the development of safer and more effective vaccines is needed.

Among the new vaccine platforms, genetic vectors, including recombinant plasmid DNA vectors, have been widely used to deliver microbial antigen-coding genes. These vectors can strongly induce both CD4^{+} and CD8^{+} T-cell responses, which are required for an effective TB vaccine [9]. Primarily due to their flexibility, pcDNA3 and its derivatives are among the most commonly used commercial mammalian expression systems. Plasmid pcDNA3 has been widely used to make vaccines [10-11] and in cancer gene therapy [12-13]. Baghani et al. (2015) designed and produced a eukaryotic expression vector encoding *M. tuberculosis* using a pcDNA3.1 vector coding map [14]. Samira et al. (2016) also used a pcDNA3.1 vector to design a DNA vaccine encoding the *tb10.4* gene from *M. tuberculosis* [9].

The development of an effective vaccine against *M. tuberculosis* is a top priority for reducing TB morbidity and mortality. To produce new TB vaccines, several strategies have been used, including the development of DNA vaccines encoding antigens from *Mycobacteria*. Most evidence suggests that the antigens secreted by *M. tuberculosis* may stimulate protective immunity [15,16].

In the development of new TB vaccines, the resuscitation promoting factor (Rpf) family of proteins is considered a promising group of target antigens [17,18]. *M. tuberculosis* encodes five Rpf proteins: RpfA (Rv0867c), RpfB (Rv1009), RpfC (Rv1884c), RpfD (Rv2389c), and RpfE (Rv2450c) [19]. A number of studies have investigated the immune response to Rpf. A mouse infection study showed that RpfA, RpfB, RpfD, and RpfE were immunogenic [17-18]. It has also been demonstrated that the Rpf antigen induces the

production of IFN-γ by CD4^{+} T-cells [20]. More recent studies have shown that RpfA and RpfD are recognized by CD4^{+} and CD8^{+} T-cells from healthy individuals who have latent infections characterized by cytokine production against these antigens [21].

In the present study, we used one of the five Rpf family proteins encoded by *M. tuberculosis*: RpfD. All proteins in the Rpf family are able to stimulate the antibody response, stimulate T-cell proliferation, and modulate the immune response during infection in mice [18]. The *rpfD* gene is 465 bp in length and encodes a protein containing 154 amino acids. The RpfA and RpfD proteins have also been shown to be immunogenic in humans, based on their production of specific cytokines [22].

The aim of this study was to clone the *rpfD* gene of the *M. tuberculosis* Beijing strain from Indonesian patients into the mammalian expression vector pcDNA3.1(+). This recombinant plasmid is expected to induce immunity in animal models and is considered to be a new candidate for a TB vaccine.

Materials and Methods

DNA Extraction. *M. tuberculosis* Beijing strain from Indonesian patients was isolated using a PeqGOLD Bacterial DNA Kit (PeqLab) and kindly donated from Pusat Biomedis dan Teknologi Kesehatan Kementrian Kesehatan Republik Indonesia.

Amplification of the *rpfD* gene. The *rpfD* gene was amplified using PCR with specific, proprietary primers annealing to the *rpfD* region. The PCR reaction mixture contained the following ingredients: 50 pmol of forward primer and reverse primer for *rpfD*; 5 μL of *M. tuberculosis* genomic DNA; 10 mM of dNTPs; 50 mM of $MgCl_2$; and 1 U/μL of Phusion Hot Start II DNA Polymerase (Thermo Fisher Scientific). The final reaction volume was 50 μL. The first cycle of the PCR reaction was performed for 5 min at 98 °C, followed by 30 cycles of 30 sec at 98 °C, 30 sec at 58 °C, and 1 min at 72 °C. The final cycle was performed for 7 min at 72 °C. The PCR product size was confirmed by gel electrophoresis and then purified using a GeneJET PCR Purification Kit (Thermo Fisher Scientific).

rpfD cloning into vector pcDNA3.1(+). The PCR product and plasmid pcDNA3.1(+) were digested using *Eco*RI (BioLabs) and *Hin*dIII (Thermo Fisher Scientific). These enzymes have restriction sites within pcDNA3.1(+). The reaction was performed twice for each enzyme with a specific buffer and condition. Then, the digested products were purified using a GeneJET PCR Purification Kit for the PCR fragments and a GeneJET Plasmid Miniprep Kit (Thermo Fisher Scientific) for the pcDNA3.1(+). Ligation of digested *rpfD*

into pcDNA3.1(+) vector was performed using T4 DNA Ligase (Thermo Fisher Scientific).

Escherichia coli (*E. coli*) DH5α was treated with 0.05 M of cold CaCl$_2$ solution to make bacteria ready to accept foreign DNA. This strain was selected because it is appropriate for cloning. Competent cells were transformed using the heat-shock method [23]. The transformed bacteria were cultured on lysogeny broth (LB) agar containing ampicillin at a concentration of 100 μg/mL. Colony PCR was performed to identify colonies containing the recombinant vector, which was then extracted using a GeneJET Plasmid Miniprep Kit (Thermo Fisher Scientific). The accuracy of the cloning was confirmed using enzyme digestion with *Stu*I (Thermo Fisher Scientific), followed by sequencing (BioEdit software).

Results and Discussion

We amplified *rpfD* from the *M. tuberculosis* Beijing strain from Indonesia patients using PCR. A 465-bp fragment was obtained following gel electrophoresis

using 1.5% agarose (Figure 1). The genetic background of various *M. tuberculosis* strains may affect the induction of an immune response, drug resistance patterns, clinical features, epidemiology, and pathogenic characteristics that determine disease progression after infection [24]. The use of the Beijing strain from Indonesian patients was based on the results of prior studies that suggested that most of the isolates of *M. tuberculosis* in Indonesia are from the Beijing strain [25,26]. The use of the Beijing strain in vaccine development is expected to support the discovery of vaccine candidates that can be produced in Indonesia.

After digestion using *Eco*RI and *Hin*dIII, the PCR product showed no significant change in size, and the pcDNA3.1(+) vector demonstrated a 5387-bp fragment (Figure 2).

In the next step, the target gene was inserted into the backbone using T4 DNA Ligase. The ligation product was used to transform *E. coli* DH5α and yielded 100 colonies. Colony PCR was performed using the forward and reverse primers for *rpfD* to confirm gene insertion
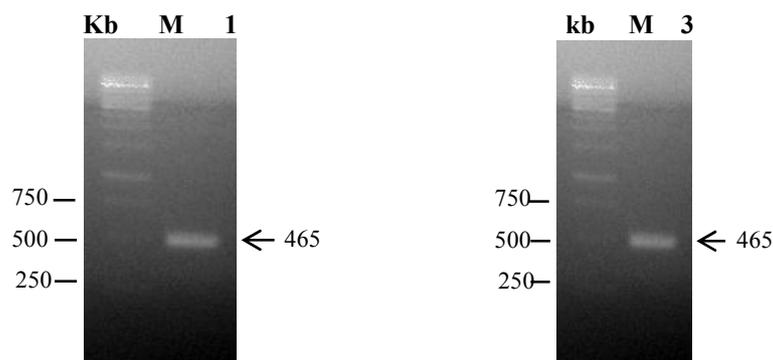

Figure 1. **Amplification, Digestion, and Purification of *rpfD* Gene-Coding Region. kb: kilobase; Lane M: 1-kb DNA Ladder; Lane 1: PCR Product of the *rpfD* Gene-coding Region; Lane 2: *rpfD* Gene-Coding Region Digested Using *Eco*RI and *Hin*dIII; Lane 3: *rpfD* Gene-Coding Region After Digestion and Purification. The Black Arrow Shows A Band that Correlates to the Length of the *rpfD***
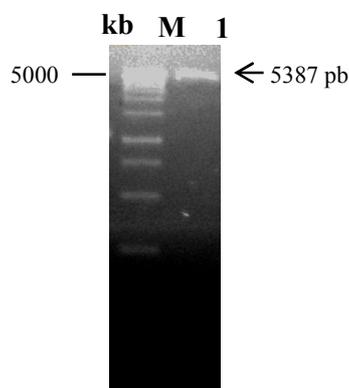

Figure 2. **Digestion of the pcDNA3.1(+) Vector Using Restriction Enzymes. kb: Kilobase, Lane M: 1-kb DNA Ladder; Lane 1: pcDNA3.1(+) Digested Using *Eco*RI and *Hin*dIII. The Black Arrow Shows A Band that Correlates to the Length of the Wild-Type pcDNA3.1(+) Vector**

into the pcDNA3.1(+) in 28 colonies that were chosen randomly. The colony PCR product was gel electrophoresed, and a 465-bp fragment was observed (Figure 3), indicating that the *rpfD* gene was successfully inserted into the pcDNA3.1(+) plasmid.

The vector used in this study, pcDNA3.1(+), uses a cytomegalovirus (CMV) promoter to express the *rpfD* gene in mammalian cell lines. This CMV promoter produces a higher transgene expression in tissues than all other promoters and can increase the expression level of recombinant proteins in various cell types [27,28]. In addition, pcDNA3.1(+) contains pUCori, which facilitates replication in bacterial cells, as well as bovine growth hormone (BGH) polyadenylation-signal and transcription-termination sequences, which function to increase the stability of the mRNA [29,30]. These features have made pcDNA3.1(+) plasmid a common choice for use

in the development of TB vaccines. Although pcDNA3.1 vector may have a different protein character than *rpfD*, some genes have successfully been cloned into it, including *cfp10*, *ag85a*, and *tb10.4* [31,32], suggesting that pcDNA3.1(+) is a suitable vector for cloning *M. tuberculosis* genes.

Five colony PCR products were selected, purified using a GeneJET PCR Purification Kit, and then digested using *Stu*I, a restriction enzyme that digests pcDNA3.1(+) vector on base 2053. The results showed that all colonies had a larger digest product than the pcDNA3.1(+) wild-type control (Figure 4). The accuracy of the cloning was confirmed using sequencing (BioEdit software), the sequencing results demonstrated that the *rpfD* DNA sequence was appropriate when compared with the sequences of the *M. tuberculosis* H37Rv strain, and no mutations were found in it.
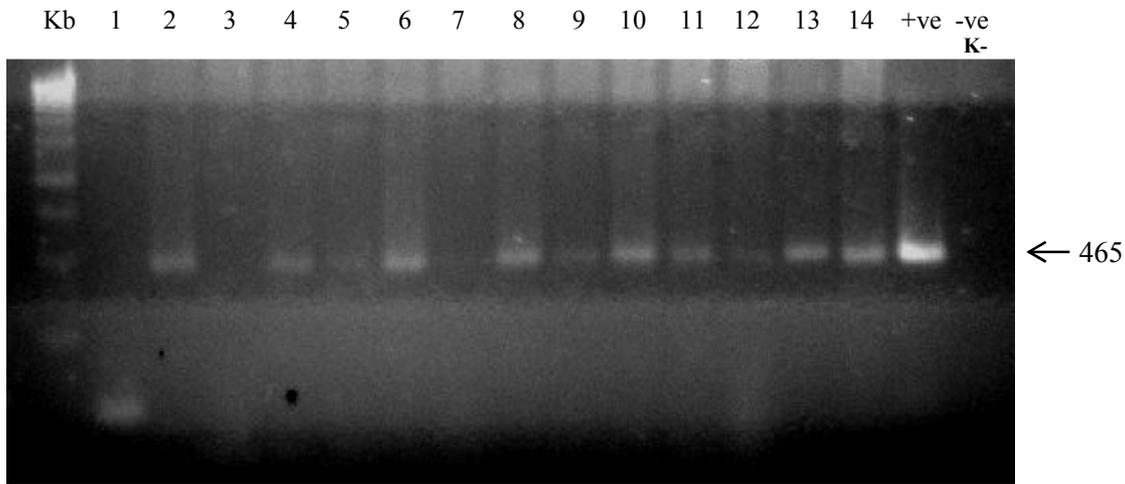

Figure 3. Colony PCR to Grow Colonies on Selective Medium After DNA Recombinant Transformation. kb: kilobase; M: 1-kb DNA ladder; Lanes 1-14: Colony PCR from *E. coli* Transformed Using pcDNA3.1(+)-*rpfD*; +ve: Positive Control Amplification of *rpfD* from *M. tuberculosis* H37Rv; -ve: Negative Control Amplification of *rpfD* from pcDNA3.1(+) Wild-Type Template. The Black Arrow Shows A Band that Correlates to Length of the pcDNA3.1(+) Vector
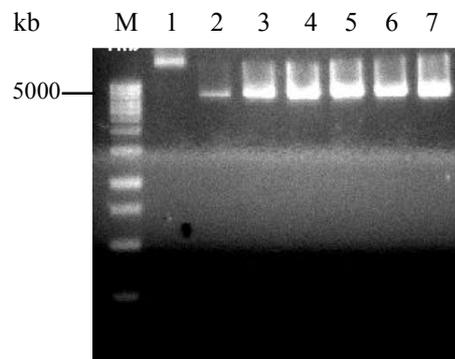

Figure 4. Confirmation of *rpfD* Insertion Into the pCDNA3.1(+) Vector Using Restriction Enzyme Digestion. kb: Kilobase; M: 1-kb DNA Ladder; Lane 1: pcDNA3.1(+) Without Digestion; Lane 2: pcDNA3.1(+) Digested Using *Stu*I; Lanes 3–7: Colonies Transformed with pcDNA3.1(+)-*rpfD* Digested Using *Stu*I

Several studies have shown that RpfD can induce a strong cellular immune response. One recent study evaluated the efficacy of a DNA vaccine containing *rpfD* that was injected into mice. It successfully induced a cellular immune response, as indicated by IFN-γ production [17]. It has also been demonstrated other Rpf family proteins like RpfA, RpfB, and RpfE are immunogenic in mice and can provide protection via humoral and cellular immune responses [18]. We propose to expand this study by expressing RpfD protein and investigating its immunogenicity in a mouse model.

Conclusions

In the present study, we successfully constructed a pcDNA3.1(+) vector encoding *rpfD* from *M. tuberculosis* for use as a vaccine antigen. Future studies will probe the RpfD protein-induced production of IFN-γ IL-12 and IgG in animal models, which may lead to promising results for human administration.

Acknowledgments

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