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by

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Ligation, Transformation and Characterization of Rv 1984c *Mycobacterium tuberculosis* Indonesian Isolate as an Antigen for Latent TB Immunodiagnostic

Rosana Agus^{1*}, Muhammad Nasrum Massi² and Zaraswati Dwyana¹

¹Department of Biology, Faculty of Life Sciences, Hasanuddin University, Indonesia

²Department of Microbiology, Faculty of Medicine, Hasanuddin University, Indonesia

*Corresponding author

Abstract

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Latent tuberculosis infection (LTBI) is characterised by the presence of immune responses to *Mycobacterium tuberculosis* infection without clinical evidence of active Tuberculosis (TB). The challenge of identifying LTBI-infected individuals lies in the lack of a diagnostic gold standard for LTBI. Tuberculin skin test (TST) has been used for the diagnosis of tuberculosis for more than a century. Therefore TST has many limitations, so it is extremely urgent to develop a diagnose method to detect LTBI accurately. In this study we used, Culture Fitrates Protein 21 (CFP 21) encoded by the Rv1984c gene is an immunodominant protein and induces IFN- γ from TB patients. The purpose of this study was to ligate, transform and characterize the Rv1984 from *M.tuberculosis* isolates Indonesia to *Escherichia coli* JM109. The study was carried out by amplifying the Rv 1984c gene with PCR, ligation to pGEM-T vector and transformation to host cell *Escherichia coli* JM 109 on LB media induced by X-gal and IPTG. Characterization of pGEM-T-Rv 1984c recombinant clones was performed with PCR and sequencing. The results obtained that the DNA inserts that are ligated to the cloning vector is true Rv 1984 c

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Introduction

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One-third of the world's population is estimated to have LTBI. They do not have active TB disease but may develop it in the near or remote future (WHO, 2015). Studies suggest that active tuberculosis will develop in 5 to 15% of persons with latent infection during their lifetimes (Getahun *et al.*, 2015).

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Identifying persons with LTBI is important to the goal of TB control and elimination because treatment of LTBI can prevent infected persons from developing TB disease and stop the further spread of TB (CDC, 2013). The diagnostic tests used to identify individuals latently

infected with *M. tuberculosis* are the in vivo tuberculin skin test (TST) and the ex vivo interferon-gamma release assays (IGRAs), are designed to identify an adaptive immune response (Mack *et al.*, 2009). Tuberculin skin tests (TST) have been the traditional method to test for LTBI (Maine CDC, 2012).

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Persons with LTBI have negative bacteriological tests: the diagnosis is based on a positive result of either a skin (TST) or blood (IGRA) test indicating an immune response to *M.tuberculosis*. Either TST or IGRAs can be used to identify candidates to LTBI treatment in high and upper-middle-income countries with estimated TB incidence less than 100,000. IGRAs should not replace

TST in low and other middle-income countries (WHO, 2015).

Therefore the specificity of PPD is questionable especially in endemic areas such as Indonesia. Given the limitations of TST tests, current research is directed to find specific antigens that will be used as immunodiagnosics.

CFP-21 is encoded in the RD2 region of *M. tuberculosis* genome, a region reported to be absent from several strains of BCG. It can elicit a strong skin test reaction, and has been broadly recognised in genetically different strains of inbred mice. CFP-21 is immunologically very active and induces a high interferon- γ (IFN- γ) release from murine memory effect cells or a pronounced DTH reaction (Wang *et al.*, 2005). CFP-21 is broadly recognised in animals of different major histocompatibility complex class II compositions (Welding *et al.*, 1998)

Materials and Methods

Bacterial Strains and Plasmids

The cloning vectors pGEM-T Easy (Promega) was used. The strains *M. tuberculosis* was obtained from clinical isolate from Indonesia (Agus, 2016).

Culture Condition

The *E. coli* JM 109 were incubate with stirring over night in Luria Bertani (LB) medium in the presence of ampicillin (1 μ g/ml) at 37°C. *E.coli* JM109 competent cells prepared in advance by the CaCl₂ methods.

PCR

The following primer was design by (Fu *et al.*, 2009)

Forward 5'-AAGGATCCGATCCGTGTTCCGACA TCGCGGTCG 3'

Reverse 5'-GCTGCCGCCACCGCCGCTTCCGCC ACCGCCGCTTCCACCGCCACCTCCGGCGTGATC GAGCCTGTTCGCC -3'

PCR reactions were performed with 1 μ l of template in a total volume of 25 μ l. Generally, the PCR profiles consisted of an initial denaturation at 94°C for 5 minute, a denaturation at 94°C for 1 minute, an annealing at 60°C for 1 minute and an extension step at 72°C for 45

second. After 30cycles an extension was carried out at 72°C for 5 minutes. PCR products were examined by agarose gel electrophoresis and staining with ethidium bromide.

Transformation

Isolation of DNA and transformation of the *E. coli* JM 109 cells were performed as described in the guidebook (Sambrook, 2001), with the following modifications. Transformed cells were spread on the appropriate indicator plates containing ampicillin. Colonies were scored for phenotype on Luria Bertani agar plates after 24 hours at 37°C.

Construction of Recombinant Plasmid of the pGEM-T-Rv 1984c in *E. coli* JM 109

Fragment of the Rv 1984c were obtained by PCR using two primers pair and DNA of the *M. tuberculosis* Indonesian strain as a template. The pGEM-T vector and PCR product were cut with *Bam*HI and *Hind*III, mixed, and treated with T4 DNA ligase. The result recombinant plasmid pGEM-T-Rv1984c was transformed in *E. coli* JM 109

Characterization of Recombinant Plasmid of pGEM-T-Rv 1984c

Isolation of plasmid were performed according to the procedure instructions (BioRad). Characterization was done by PCR analysis and sequencing. PCR analysis was done by using exactly the same cycle as it mentioned before for amplified the Rv 1984c gene

Results and Discussion

PCR Product and Purification

The results of PCR amplification of Rv 1984c on *M. tuberculosis* H37RV as positive control and clinical isolates obtained band 608 bp. It is appropriate that obtained Fu *et al.*, 2009 that 608 bp band was observed upon staining with ethidium bromide.

Transformation

The result of the transformation was tested by grown on a solid LB medium with ampicillin, X-GAL and IPTG added. Only *E. coli* clones containing recombinant clones of pGEM-T-Rv 1984c can grow. White colonies indicate that the insert DNA (Rv 1984c) has been

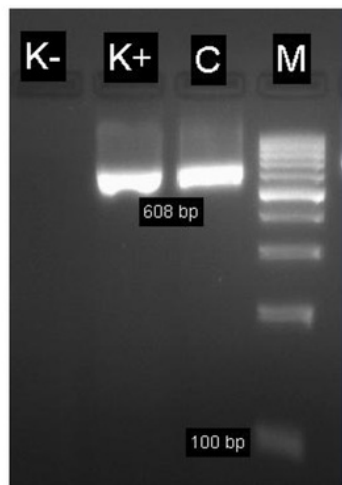
successfully inserted into the vector and the blue colonies means the DNA insertion is not successfully ligated into the vector.

1 Bacterial cells are plated on a selective agar medium containing the antibiotic ampicillin and X-gal. If foreign DNA is inserted into the multiple cloning site, then the *lacZ'* coding region is disrupted and the N-terminal portion of β -galactosidase is not produced. Since there is no functional β -galactosidase in the bacteria, the

substrate X-gal remains colorless, and the bacterial colony containing recombinant plasmid DNA appears white, thus allowing the direct identification of colonies carrying cloned DNA inserts. If there is no insertion of foreign DNA in the multiple cloning site, then the *lacZ'* gene is intact and enzymatically active β -galactosidase is produced and X-gal is degraded.

The bacterial colonies containing non-recombinant plasmid DNA thus appear blue (Dubey, 2016).

Fig.1 PCR product of Rv 1984C.



K -, K +, C, M = Negative control, Positive Control, Sample, Marker

Fig.2 Transformation of Rv 1984c gene to pGEM-T vector (1 = white colony, 2 = blue colony)

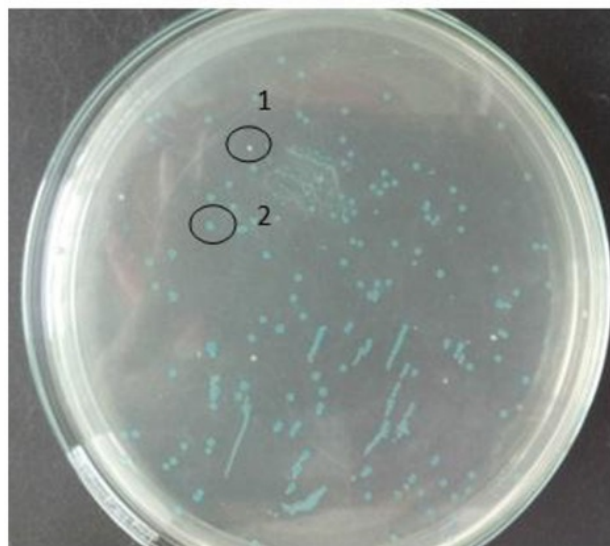


Fig.3 PCR analysis : 1, 2, 3, 4) Rv 1984c, M) Marker 100 bp

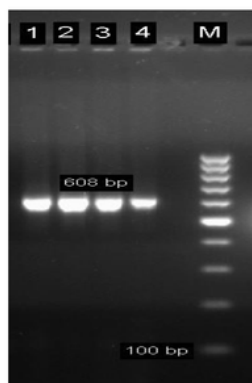


Fig.4 BLAST Analysis of gene Rv 1984c

Score	Expect	Identities	Gap	Strand
1208 bits(654)	0.0	654/654(100%)	7 0/654(0%) Plus/Plus	
Query	1	TCATCCGGCGTGATCGAGCCTGTTGCGCCGCAATGTCGCCGCCTGGCTTGTTCATCCCCGA		60
Sbjct	1	TCATCCGGCGTGATCGAGCCTGTTGCGCCGCAATGTCGCCGCCTGGCTTGTTCATCCCCGA		60
Query	61	CTGAACATACGAAACATGCGCCATAATATTGCCGCCTCCGGTGCATATTGGATCGTCGGG		120
Sbjct	61	CTGAACATACGAAACATGCGCCATAATATTGCCGCCTCCGGTGCATATTGGATCGTCGGG		120
Query	121	AGCACACAAGTTTATGGTCTTAGAGCTATACAGCGGACCGATTGTTCGGCAACGACCCGCC		180
Sbjct	121	AGCACACAAGTTTATGGTCTTAGAGCTATACAGCGGACCGATTGTTCGGCAACGACCCGCC		180
Query	181	GCCCCACAACATGCTGGAGAAACCACTGGATGGCTCGCCGAAAAGGGCGACAGCGCGCAC		240
Sbjct	181	GCCCCACAACATGCTGGAGAAACCACTGGATGGCTCGCCGAAAAGGGCGACAGCGCGCAC		240
Query	241	ATGATCTGCCACCGCGGGCGGCATCGCCGAGGTGGACAAATCGATGACCGTCGCACCCCTG		300
Sbjct	241	ATGATCTGCCACCGCGGGCGGCATCGCCGAGGTGGACAAATCGATGACCGTCGCACCCCTG		300
Query	301	CGAATAGCCACCAAGCACAATCCTGGTGTTCGGGCAGCTGGCGACGGTGCGCTGGATGTG		360
Sbjct	301	CGAATAGCCACCAAGCACAATCCTGGTGTTCGGGCAGCTGGCGACGGTGCGCTGGATGTG		360
Query	361	GGCGCTCGCATCATCGAACCCTTTGACGCGCTCGCGGGTAGTCGTCGCTTGTGGGTA		420
Sbjct	361	GGCGCTCGCATCATCGAACCCTTTGACGCGCTCGCGGGTAGTCGTCGCTTGTGGGTA		420
Query	421	GTTACCCGCTAGACCCCAATCGACCGCCCGCCAACCTTGCAGGTAAGCGAGTCGACGAA		480
Sbjct	421	GTTACCCGCTAGACCCCAATCGACCGCCCGCCAACCTTGCAGGTAAGCGAGTCGACGAA		480
Query	481	CGCCTCACCGACGTGCGCAAGACCAGAAGCCTGATGCGTGCCTGCGAGCGAAAACGACCGC		540
Sbjct	481	CGCCTCACCGACGTGCGCAAGACCAGAAGCCTGATGCGTGCCTGCGAGCGAAAACGACCGC		540
Query	541	GATGTCCGAACACGGATCCGCATGCGCGGCACGACCGCCGGCGGGTGCCTCACCAGCGC		600
Sbjct	541	GATGTCCGAACACGGATCCGCATGCGCGGCACGACCGCCGGCGGGTGCCTCACCAGCGC		600
Query	601	CAAGGTCGTGCAACCACGACACCAACGATGCGAACAAGGCTGCGTGGAGTCAT		654
Sbjct	601	CAAGGTCGTGCAACCACGACACCAACGATGCGAACAAGGCTGCGTGGAGTCAT		654

Characterization of Recombinant Plasmid of pGEM-T-Rv 1984c

Characterization of recombinant plasmid was done by PCR analysis and sequencing. The plasmid isolation was performed on white colonies and PCR was done by using the same cycle as it mentioned before for amplified the Rv 1984c gene. Electrophoresis showed that plasmid recombinant contain the Rv 1984c gene as DNA insert was 608 bp (Figure 3)

Sequencing

Results sequencing of genes Rv 1984c with BLAST results obtained 100% homology (Figure 4). This suggests that the DNA inserts that are ligated to the pGEM-T vector is true Rv 1984c.

Ligation Rv 1984c to pGEM-T vector and transformation to *E.coli* JM 109 obtained recombinant plasmid pGEM-T-Rv1984c. After characterization with PCR and sequencing it was found that the genes had been ligated and transformed were true Rv 1984c

Acknowledgments

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