Int.J.Curr.Res.Aca.Rev.2017; 5(9): 39-43

EXCELLENT PUBLISHERS

International Journal of Current Research and Academic Review

ISSN: 2347-3215 (Online) ⁘ Volume 5 ⁘ Number 9 (September-2017)

Journal homepage: http://www.ijcrar.com

doi: https://doi.org/10.20546/ijcrar.2017.509.006

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

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Abstract

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 Fitrate 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 *Eschericia 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

Article Info

Accepted: 28August 2017
Available Online: 20 September 2017

Keywords

Culture Fitrate Protein 21,
Rv 1984c,
Ligation,
E. coli JM 109

Introduction

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).

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).

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 immunodiagnostics.

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- gamma (IFN-λ) 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 (Weldingh *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 (1µg/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'-AAGGATCCGATCCGTGTTCGGACA TCGCGGTCG 3'

Reverse 5'-GCTGCCGCCACCGCCGCTTCCGCC ACCGCCGCTTCCACCGCCACCTCCGGCGTGATC GAGCCTGTTCGCC -3'

The PCR reactions were performed with 1 µl of template in a total volume of 25 µl. 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 sequensing. 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

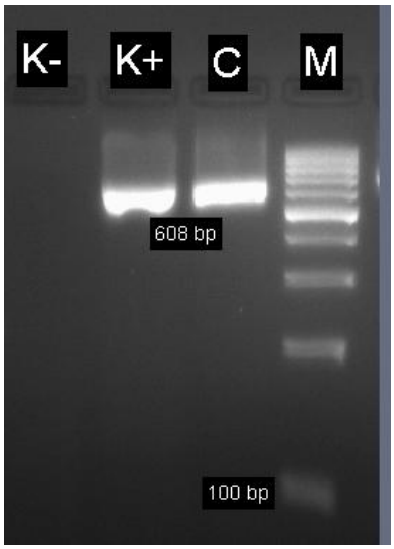
The result of the transformation was tested by grown on a solid LB medium with ampicillin, X-GAL and IPTG added. Only *E. coli* clones containing recombinant clones of pGEM-T-Rv 1984c can grow. White colonies indicate that the insert DNA (Rv 1984c) has been

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

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

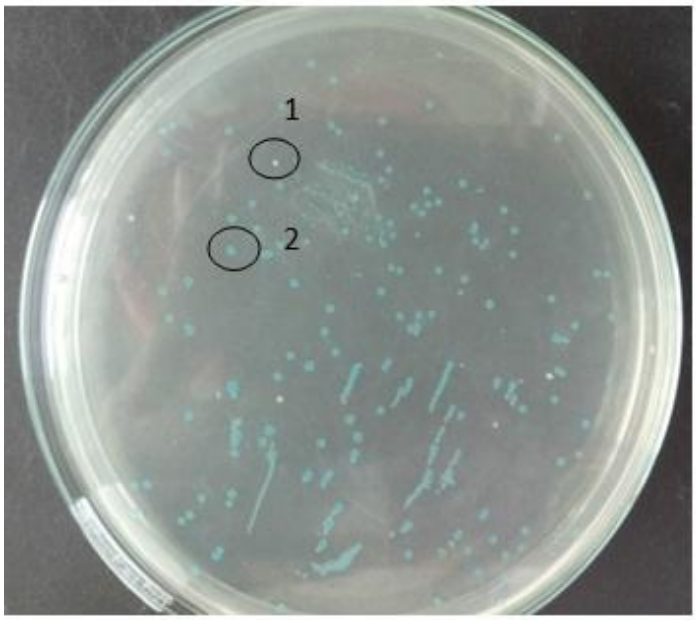


Fig.3 PCR analysis : 1, 2, 3, 4) Rv 1984c, M) Marker 100 bp

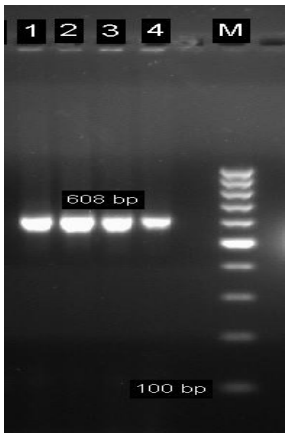


Fig.4 BLAST Analysis of gene Rv 1984c

Score	Expect	Identities	Gaps	Strand
1208 bits(654)	0.0	654/654(100%)	0/654(0%)	Plus/Plus

```
Query  1    TCATCCGGCGTGATCGAGCCTGTTCGCCGCGAATGTCGCCGCCTGGCTTGTCATCCCCGA  60
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1    TCATCCGGCGTGATCGAGCCTGTTCGCCGCGAATGTCGCCGCCTGGCTTGTCATCCCCGA  60

Query  61   CTGAACATACGAAACATGCGCCATAATATTGCCGCCTCCGGTGCATATTGGATCGTCGGG  120
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  61   CTGAACATACGAAACATGCGCCATAATATTGCCGCCTCCGGTGCATATTGGATCGTCGGG  120

Query  121  AGCACACAAGTTTATGGTCTTAGAGCTATACAGCGGACCGATTGTCGGCAACGACCCGCC  180
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  121  AGCACACAAGTTTATGGTCTTAGAGCTATACAGCGGACCGATTGTCGGCAACGACCCGCC  180

Query  181  GCCCCACAACATGCTGGAGAAACCACTGGATGGCTCGCCGAAAAGGGCGACAGCGGCGAC  240
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  181  GCCCCACAACATGCTGGAGAAACCACTGGATGGCTCGCCGAAAAGGGCGACAGCGGCGAC  240

Query  241  ATGATCTGCCACCGCGGGCGGCATCGCCGAGGTGGACAAATCGATGACCGTCGCACCCTG  300
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  241  ATGATCTGCCACCGCGGGCGGCATCGCCGAGGTGGACAAATCGATGACCGTCGCACCCTG  300

Query  301  CGAATAGCCACCAAGCACAATCCTGGTGTTCGGGCAGCTGGCGACGGTGCGCTGGATGTG  360
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  301  CGAATAGCCACCAAGCACAATCCTGGTGTTCGGGCAGCTGGCGACGGTGCGCTGGATGTG  360

Query  361  GGCGCTCGCATCATCGGAACCGTTTGACGCGCTCGCGCGGTAGTCGTCGCTTGCTGGGTA  420
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  361  GGCGCTCGCATCATCGGAACCGTTTGACGCGCTCGCGCGGTAGTCGTCGCTTGCTGGGTA  420

Query  421  GTTCACCGCGTAGACCCCAATCGACCGCCCGCCAACTTGCGAGGTAAGCGAGTCGACGAA  480
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  421  GTTCACCGCGTAGACCCCAATCGACCGCCCGCCAACTTGCGAGGTAAGCGAGTCGACGAA  480

Query  481  CGCCTCACCGACGTCGCCAAGACCAGAAGCCTGATGCGTGCCGCGAGCGAAAACGACCGC  540
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  481  CGCCTCACCGACGTCGCCAAGACCAGAAGCCTGATGCGTGCCGCGAGCGAAAACGACCGC  540

Query  541  GATGTCCGAACACGGATCCGCATGCGCGGCACGACCGCCGGCGGGTGCGCTCACCAGCGC  600
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  541  GATGTCCGAACACGGATCCGCATGCGCGGCACGACCGCCGGCGGGTGCGCTCACCAGCGC  600

Query  601  CAAGGTCGTCGCAACCACGACACCAACGATGCGAACAAGGCTGCGTGGAGTCAT  654
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  601  CAAGGTCGTCGCAACCACGACACCAACGATGCGAACAAGGCTGCGTGGAGTCAT  654
```

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

Characterization of recombinant plasmid was done by PCR analysis and sequensing. 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 sequensing 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

The authors would like to thank Kemenristek Dikti who has funded this research through Hibah Kompetensi 2017

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How to cite this article:

Rosana Agus, Muhammad Nasrum Massi and Zaraswati Dwyana. 2017. Ligation, Transformation and Characterization of Rv 1984c *Mycobacterium tuberculosis* Indonesian Isolate as an Antigen for Latent TB Immunodiagnostic. *Int.J.Curr.Res.Aca.Rev.* 5(9), 39-43. **doi:** https://doi.org/10.20546/ijcrar.2017.509.006