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CLONING OF ANTIGEN 85A ISOLATED FROM MYCOBACTERIUM TUBERCULOSIS INTO *E.COLI* DH5A HOST

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ABSTRACT

Previous studies of antibody responses to *Mycobacterium tuberculosis* antigens at different stages of tuberculosis (TB) and in different classes of TB patients provided evidence that the profile of antigens recognized by antibodies is altered with disease progression. M. tuberculosis is a slow-growing organism, and it takes several weeks to months for an infection to progress to clinical TB. Approaching into the antigens expressed in vivo during subclinical TB could contribute to our understanding of the host pathogen interaction that leads to progression to clinical TB. Many amplification targets for *M. tuberculosis* have been reported. One of the more common PCR targets is the antigen 85A, specific for *M. tuberculosis* complex. The proteins of the antigen 85 complex are major secretion products of *Mycobacterium tuberculosis* and *Mycobacterium bovis* and have been studied by independent investigators for at least three decades. In the present study mycobacterium tuberculosis bacteria were isolated from patient with pulmonary tuberculosis and using specific primer designed using Primer 3 plus software for the Mycobacterial antigen 85A gene. The amplified gene was ligated with T vector (pTZ57R/T) and transformed into DH5α cells. The plasmid DNA obtained was then confirmed by restriction digestion and sequence analysis. The sequence was found to be 98% similar to that obtained in GenBank. Dendrogram was constructed using ClustalW software to get the similarity of the sequence with the existing sequence in the NCBI. Further research is required to express the gene to get the protein antigen for the production antibodies or effective vaccine for *Mycobacterium tuberculosis*.

KEYWORDS: ***Mycobacterium tuberculosis*, Genomic DNA, PCR, Cloning, Antigen 85A**

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INTRODUCTION

Although, it is well known that the members of the Ag85 family are found in all mycobacteria, sequence comparison shows that the Ag85 gene family arose by primordial duplications of an familial gene, almost certainly before the emergence of the known mycobacterial species. Genetic disruption of these genes encoding the three Ag85 components of *M. tuberculosis* implies that Ag85A potentially the most vital component for bacterial survival within macrophages[1, 2]. Ag85A induces enhanced T-cell proliferation and gamma interferon production in most healthy individuals infected with *M. tuberculosis* or *Mycobacterium leprae*[3] and in *Mycobacterium bovis* BCG-vaccinated mice[4]. Owing to the good efficacy of Ag85 components of *M. tuberculosis including* Ag85Aand Ag85B in experimental mouse and guinea pig models, they are placed among the most capable candidates for future subunit tuberculosis vaccines. Undeniably, vaccination of C57BL/6 mice with naked plasmid DNA encoding Ag85A and Ag85B can stimulate strong humoral and cell-mediated immune responses and confer significant protection against aerosol or intravenous challenge with live *M. tuberculosis* H37Rv [5, 6, 7].

As the human TB vaccine Mycobacterium bovis bacille Calmette-Guérin (BCG)[8] is only moderately effective in cattle and humans, the development of an effective vaccine protecting against bovine TB potentially afford a cost-effective TB control approach and also manage the human TB caused by Mycobacterium tuberculosis. It was found that the protective efficacy of the BCG vaccine can be increased by boosting with purified Ag85A protein. In the light of the above findings, the objective of the present study was to find if the mycobacterial fusion protein antigen 85A (Ag85A) isolated from *mycobacterium tuberculosis* could be produced in *Escherichia coli* and confirm the sequence of the protein expressed by *E.coli* with reference to the GENBANK.

MATERIALS AND METHODS

DNA Isolation and PCR

Blood sample was collected from the pulmonary tuberculosis patient from the Govt Hospital, Bangalore, India. Genomic DNA was isolated from *Mycobacterium tuberculosis* using the phenol, chloroform, and isoamyl alcohol (25:24:1) extraction method. In order to amplify the specific gene of our interest antigen 85A product, PCR was performed using the following conditions: complete denaturation: 95°C for 2 min; Annealing: 52°C for 30 sec; Extension: 72°C for 1 min, followed by 30 cycles of amplification and the final elongation step (72°C for 2 min) using Forward Primer: 5'GAGATGAGGATGAGGGAAGC3' and Reverse primer 5' CCGCCGCTAGATGTTGTGTCT 3'. PCR products were separated and analyzed on 1 % agarose gel electrophoresis.

Cloning of antigen 85A gene

The PCR product of antigen 85A gene was eluted from the gel and cloned in T vector pTZ57R/T (Fermentas, Germany) as per manufacturer's instructions. The ligated mix was then transformed into competent E. coli DH5α cells, by CaCl2 method. The transformants were plated on Luria broth (LB) agar supplemented with Ampicillin (50mg/mL), in addition with IPTG (40mg/mL), and X-gal (20mg/mL). Cells were incubated at 37º C for overnight. Blue–White screening colony selection method was performed to choose the white colored recombinant clone and followed by colony PCR amplification was performed for confirmation of cloning of our gene of interest (antigen 85A gene).

Plasmid Isolation

The plasmid was isolated from positive clones by alkali-lysis method described by Sambrook *et a l*[9]. Briefly, 2ml of Overnight culture was centrifuged. The cell pellet was resuspended in 200µl ice cold lysis solution 1 (which consist of 15% glucose, 25mM Tris,

10mM EDTA) and followed by vortexed gently. Then added 400 µl of freshly prepared solution 2 (which consist of 0.2N NaOH, 1% SDS) and 50 µl solution 3 (3M Sodium acetate). Centrifugation was done at 10,000 rpm for 10 minutes and to the supernatant equal volume of iso-propanol was added and incubated at RT for 15 min. Then centrifuged at 10000 rpm for 10 min, removed the supernatant and pellet was dissolved in 50µl TE buffer for further use**.**

Confirmation of clone by plasmid isolation and restriction digestion

The recombinant white colonies were isolated from the LB- Ampicillin agar plates and inoculated in LB Ampicillin containing broth and incubated at 37°C for overnight. Plasmid DNA was isolated by Alkaline-lysis method. The purified plasmid was subjected to restriction digestion using *Bam*H1and EcoR1. The release of the gene product was checked on 1% agarose gel electrophoresis.

RESULTS AND DISCUSSION

Genomic DNA isolation and quantification

The blood sample was incubated in LJ medium for 2-3 weeks. The small colonies developed in the plate, based on the colony morphology seven bacteria were transferred in to LJ broth and used for DNA isolation. The *Mycobacterium tuberculosis* was cultured in the LJ broth media and genomic DNA was isolated by modified CTAB method. The isolated DNA was electrophorized in 1% Agarose gel (Fig.1). The quantity and quality of DNA was analyzed by UV visible spectrophotometer (viva spec, Germany). A260/280 value of DNA samples showing the purity of the isolated DNA. The purity of DNA was indicated by A260/A280 ratio, where the value from 1.8 to 2.0 was considered as high purity. Nevertheless, the extracted DNA is considered of adequate purity if A260/A280 is > 1.5

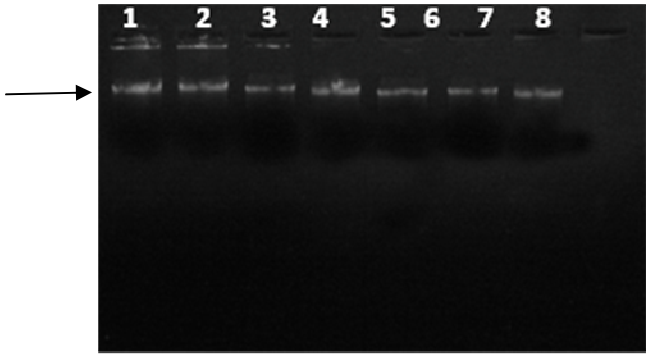

Figure 1
Genomic DNA isolated from the bacteria

PCR amplification of the *Mycobacterium tuberculosis* antigen *85A* genes

The products from the amplification of the specific PCR primers were resolved by electrophoresis in 1.5% Agarose gel and visualized by ethidium bromide staining. The primers could yield an amplicon of the expected size specific to *85A* gene. The primers were found to produce ~1070 bp amplicon which shown in the figure 2.

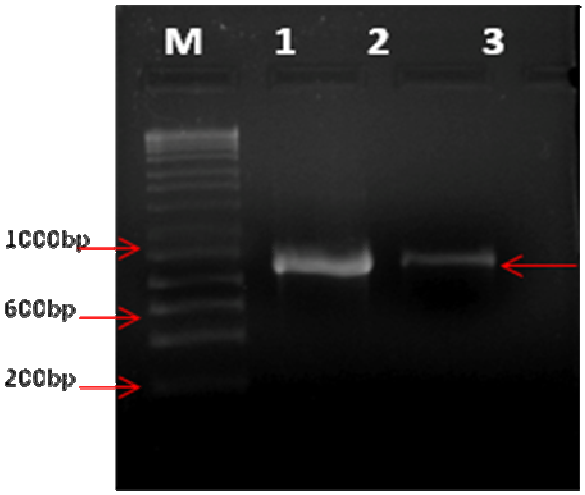

Figure 2
PCR amplification of 85A gene by specific primers (M- 200bp ladder, Lane 1, 2- 85A gene product 3-7- Negative samples)

Cloning of PCR product in to T vector

PCR yielded a specific amplicon of ~1074-bp in three of the samples (Fig. 2). The fragment was separated in gel and purified the product using gel extraction kit. The purified PCR product was quantified and ligated with cloning vector (Fig. 3) using T4 DNA ligase enzyme.

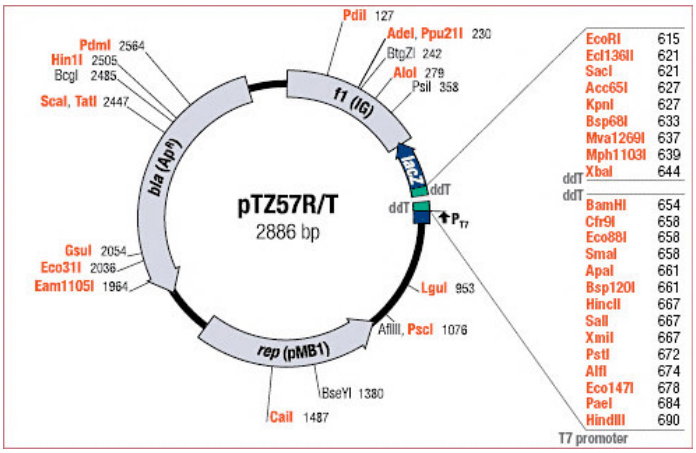

Figure 3
Map of T vector pTZ57R/T (Fermentas, Germany)

The ligated plasmid was transformed in to *E.coli* bacterial strain DH5α. The transformation was done by heat shock method and transformed cell was cultured in the Xgal-IPTG-Ampicillin-LB Agar plate at 37^{o}C for overnight (Fig. 4). The white colonies were picked up from the plates and cultured in Ampicillin containing LB broth.

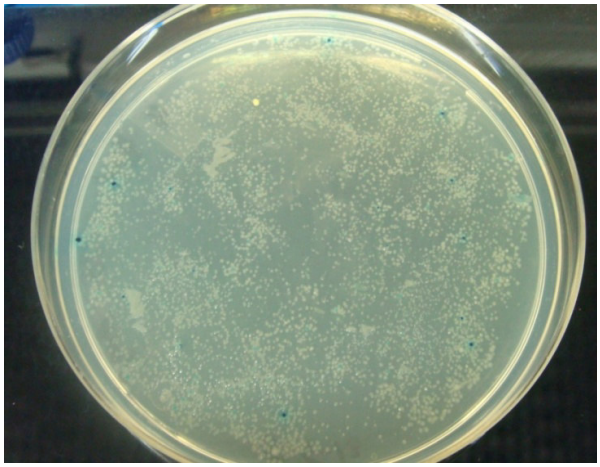

Figure 4
Blue white selection of the transformed bacterial cells in the Xgal-IPTG-Ampicillin-LB Agar.

Plasmid isolation and Confirmation of clone by restriction digestion

Plasmid was isolated from the transformed cells by using alkaline lysis method. The isolated plasmid was electrophorized on 1% Agarose gel. The purified plasmid was subjected to restriction digestion using *Bam* H1 and *Eco*R 1 (Merck, India). After incubation at 37°C for 4 hours the restricted product was electrophorized on 1% Agarose gel. The release of the gene product was visualized in the gel (Fig. 5).

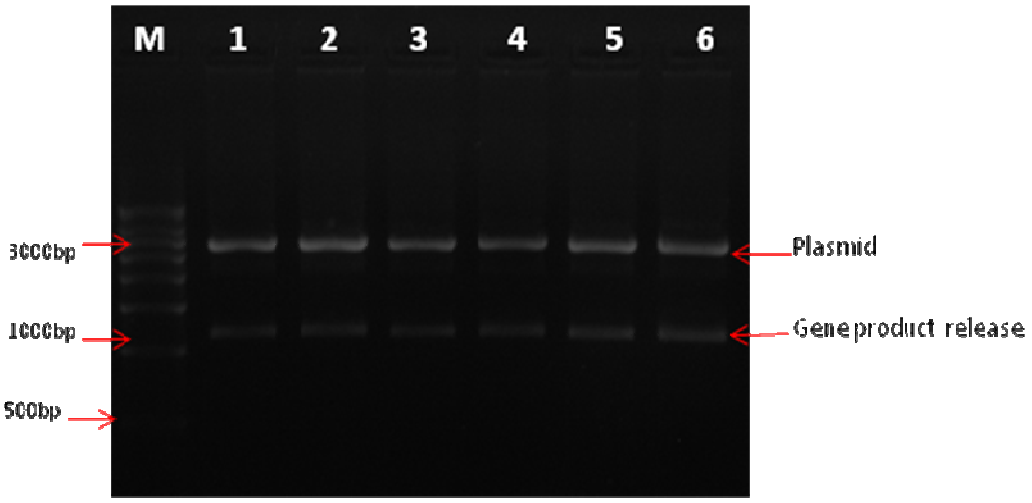

Figure 5
Restriction digestion of ligated plasmid using Bam H1 and EcoR 1 (Lane 1-6 - restricted product, M-500bp ladder)

Sequence data

The gene was identified by sequencing of plasmid. An approximately ~1074-bp region of the, antigen *85A* genes was sequenced at Eurofins, Bangalore. Nucleotide sequence analysis of gene was used to investigate the identity of bacterial antigen *85A* genes of *Mycobacterium tuberculosis.* To demonstrate the quality and accuracy of results provided from a public database, we compared sequences to their corresponding GenBank sequences. The sequence had "perfect" match (similarity, 99%) with sequences of their corresponding gene from GenBank as determined by using BLAST (version 2.7).

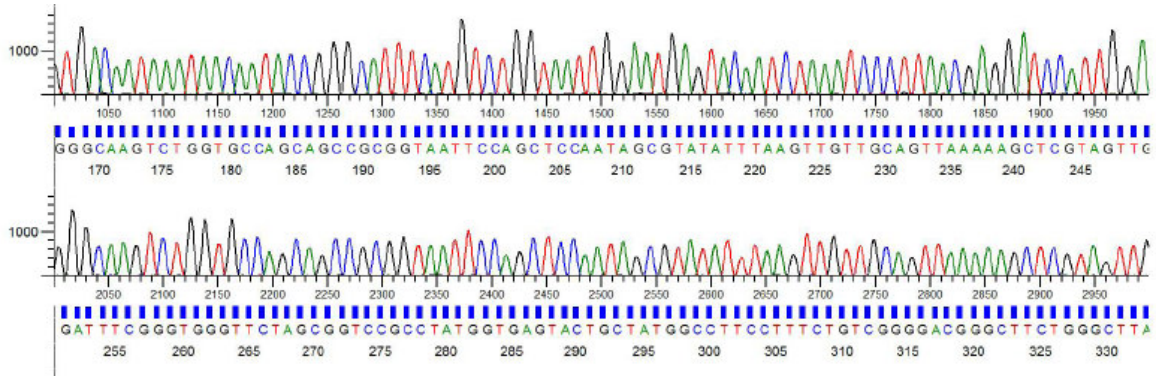

Figure 6
Showing the sequencing dendrogram

Sequence alignment by clustalW

The *85A* gene sequence of *Mycobacterium tuberculosis*is also available in GenBank database, which is more similar to this sequence (99% similarity). The N-J tree with branch length was plotted using ClustalW sequence alignment (http://align.genome.jp/), showing the relationship of *85A* gene among the closest *Mycobacterium tuberculosis*strains in the NCBI database. The sequence of *Mycobacterium tuberculosis* antigen *85A* gene amplified by the specific primer is closely matching (99%) with *Mycobacterium tuberculosis.* Sequence analysis of the *85A* gene, genes from 12 strains was also carried out to obtain new insight into the genetic differences between the different mycobacterial strains. Interestingly, we found 3 mutations within the *85A* gene open reading frame of our strains compared with previously published sequences *85A* gene. It is well known that local DNA sequences containing repeat sequences (direct repeats or inverted repeats) may cause deletion by misalignment during DNA replication or recombination. However, Huygen *et al.* (1988) found that 71% of 21 patients treated for active tuberculosis and all 12 tuberculin positive healthy volunteers responded to purified antigen 85A.

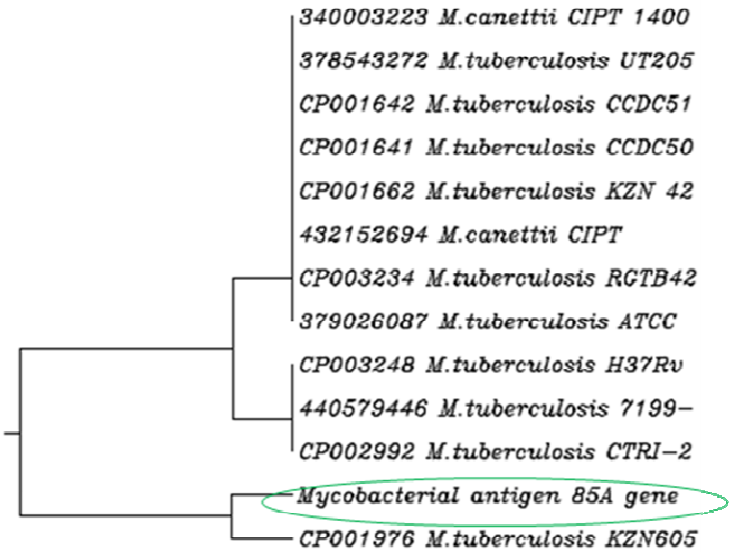

Figure 7
A tree plot was constructed with the NJ method using ~1074bp fragment of the 85A gene partial sequences showing the relationship of Mycobacterium tuberculosis.

CONCLUSION

Insight into the antigens expressed in vivo during subclinical TB could contribute to our understanding of the host pathogen interaction that leads to progression to clinical TB Many amplification targets for *M. tuberculosis* have been reported [10]. The proteins of the antigen 85 complex are major secretion products of *Mycobacterium tuberculosis* and *Mycobacterum bovis* and have been studied by independent investigators for at least three decades. In the present study mycobacterium tuberculosis bacteria were isolated from patient with pulmonary tuberculosis and a recombinant plasmid DNA which contains the gene encoding the protein Ag85A of Mycobacterium tuberculosis in E.coli strain has been successfully constructed. The results of the present study possibly remain as a basis for further studies on the usefulness of the gene and its expression product in the development of new vaccine against tuberculosis.

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