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Cloning and sequencing of *M. tb* gene Rv0378 for making subunit based DNA vaccine

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Abstract. *Mycobacterium tuberculosis* has infected one third of the world's population and 1.5 million people die each year due to tuberculosis. The research was conducted to make clones of *M. tb* gene Rv0378 during January–November, 2015. Gene Rv0378 has a gene length 222bp and was amplified using proper sequence specific primers. The size and quality of DNA fragments were determined by agarose gel electrophoresis. PCR product was then ligated in Vector PtZ57R/T (T/A vector) in order to be transformed into competent alpha-*DH5* (*E. coli*) cells. Ampicillin positive clones were selected from the plates, introduced in the autoclaved test tubes with 2 to 3 ml. SOB broth was then placed in a shaker incubator for overnight at 37°C. Next day turbidity was clearly observed in the test tubes indicating the culture was ready to use for plasmid extraction. DNA was extracted by using Genejet Plasmid extraction miniprep Kit by Thermo scientific. Size of the extracted construct was about >3000bp (other impurities of proteins and salts are washed away in EZ-Spin columns). In next step it will be cloned into pND14 (A mammalian expression vector) to make candidate vaccine and will be tested for its efficacy against *M. tb*.

INTRODUCTION

Tuberculosis (TB) an infectious disease that can affect many parts of the body, but lungs are the preferred site of infection (WHO, 2011). TB is a disease of lower respiratory tract and inhaled bacterium can be deposited into the alveoli where it is encountered by alveolar macrophages (Li, 2008). *Mycobacterium tuberculosis* (*M. tb*) is a causative agent of TB. It is a slow growing acid fast bacillus, which usually takes 2 to 6 weeks to grow on selected media. It has a thick waxy cell wall which makes them resistant against different mechanical and chemical stress. It evades the immune system by parasitizing macrophages of its host. The high immunogenic potential of *M. tb* is based on its unusual cell envelope which is exceptionally rich in lipids, glycolipids and polysaccharides (Brulle *et al.*, 2010). Each year, 8 million new TB cases appear and 2 million individuals die of TB (Kaufmann, 2006). During the next 25 years it is anticipated that more than 40 million people may be killed by tuber-culosis, unless the control measures are implemented (Neonakis *et al.*, 2008).

Today the slow development of new antimycobacterial drugs, the need for prolonged therapy regimens, and the emergence of multidrug-resistant (MDR) and extremely drug-resistant (XDR) strains of *M. tb* are issues that highlight the reemerging TB crisis (Floyd, 2009).

BCG vaccination is linked with a decrease in mortality from other contagious diseases. Similarly BCG is less effective at preventing late reactivation of pulmonary TB. This report will evaluate the present status of works over the last years to develop a new effective vaccine than Bacille Calmette-

Guerin and highlight the challenges to apply the candidate vaccines in humans (Kristensen *et al*., 2000). In the early 1990s, DNA vaccines first sparked the interested of the scientific community when it was reported that plasmid DNA, induced antibody responses to viral and non viral antigens when delivered into the skin or muscle (Ferraro *et al*., 2011).

The present study aimed to amplify *M. tb* gene Rv 0378 and clone in T/A vector. Furthermore this gene was sub-cloned into mammalian expression vector pND to be used as DNA vaccine.

MATERIALS AND METHODS

Amplification of gene Rv0378: *M. tb* DNA was obtained from TB Diagnostic Laboratory, Mayo Hospital Lahore. Using Polymerase chain reaction, DNA sequences of Rv0378 was amplified by using following primers:

Rv0378 F AGATCTGCCACC ATGAGCGTGTATAAAGTG

Rv0378 R AAGCTT TTAGCGCGGCTGCGCCGGGCG

The PCR product was confirmed by running it on gel with GeneRuler 100bp Plus DNA Ladder (Thermo).

T/A Vector Cloning: In order to be transformed into competent cells the PCR product was then ligated by mixing PCR product (5ul), Vector PtZ57R/T (1ul), 10X ligation Buffer (4 ul) and T4 DNA ligase (1ul) in a microcentrifuge tube on ice for 30 minutes at 20C. The size of construct was confirmed by running it on gel with GeneRuler 100bp Plus DNA Ladder (Thermo). Further it was confirmed by restriction digestion by using Sal1 and BamH1 restriction enzymes.

Transformation: 200ul of alpha-*DH5* (*E. coli*) competentcells were treated with the 10 ul of ligated product by following Sambrook and Russell (2001) protocol of transformation. The transformed colonies appeared on agar plates were picked onto LB nutrient medium , which were then grown for overnight on shaker incubator at 37C.

Plasmid isolation: The protocol for Plasmid DNA purification was adapted from BIO BASIC INC. BS414-100Preps EZ-10 Spin Column Plasmid DNA Kit. This kit provides a simple and efficient method for mini plasmid DNA purification. The plasmid DNA is selectively absorbed in silica gel-based EZ-10 column and other impurities such as proteins, salts, nucleotides are washed away. Eluition of DNA was done by using kit eluition buffer. The extracted DNA was now stored at -20°C. DNA fragments were visualized and confirmed when bands of different size was run on gel and had seen on UV Compact lamp.

Gene Sequencing: The isolated construct (T/A-Rv0378) was sent to Center of Excellence in Molecular Biology (CEMB) University of The Punjab, Pakistan along with its primer set for sequencing.

RESULTS AND DISCUSSION

PCR and gene sequence

M. tb gene Rv0378 was amplified by using sequence specific primers. PCR conditions were optimized especially by changing annealing temperatures (Fig. 1a & b). Following is the gene sequence:

gtgtcgggccgctgggaggccgggaatgcggacggtaacggtggctccgcgggggttgatc
ggcagcggcgggccggcggcgacggcggtagcggcgggccaccggcgccggtggcgaa
ggtggcgatgctggagcaagcgggtccataaacggcaacgccggcgaccccggcaacagc
ggagaacgcggcgcagtgggcaagcccggcgcacccggctga

Confirmation

After getting exact sized PCR product, it was further confirmed through restriction digestion. Enzyme DnpI was used and restricted product with expected sizes of DNA was obtained (Fig. 2).

Confirmation of Rv0378 clones in T/A vector

The gene was further cloned in T/A vector (Ins TAclone PCR cloning Kit Fermentas Cat # 1213). The ampicillin positive clones were

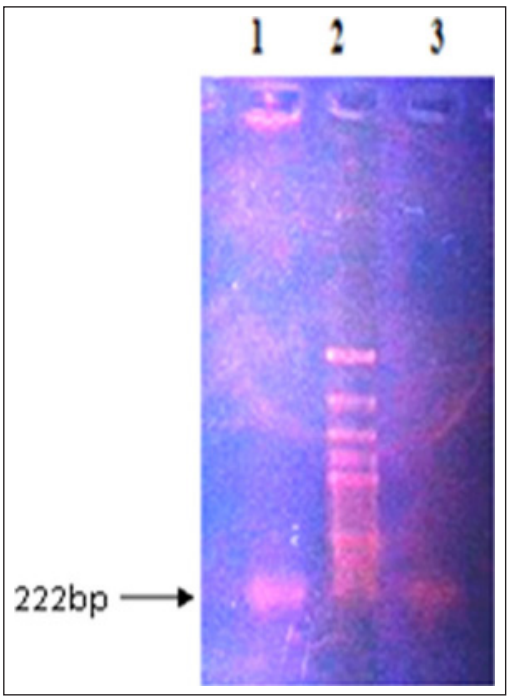

Figure 1. Lane 2: 100 bp ready to use DNA marker (Cat = SM0323 Thermo scientific). Lane 1 and 3: Amplification of *M. tb* gene Rv0378 at 58°C.

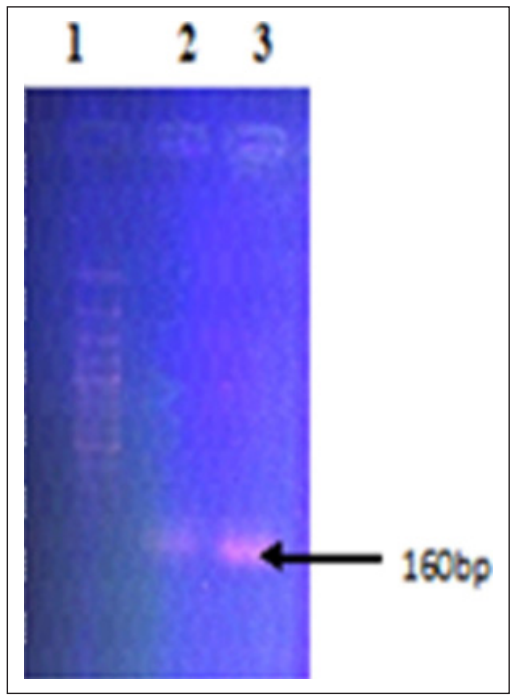

Figure 2. Restriction digestion of T/A-Rv0378 gene. Lane 1: 100 bp ready to use DNA marker (Cat = SM0323 Thermo scientific). Lane 2–3: Digestion of T/ARv0378 gene with BamH1 and SalI.

selected from plates and DNA was extracted. The expected size of positive T/A clone was >3000 bp (Fig. 3).

Tuberculosis is caused by intercellular pathogen *Mycobacterium tuberculosis*, the leading cause of infectious death. The increasing prevalence of multidrug-resistant (MDR) and even extensively drug-resistant (XDR) *M. tb* strains, and the rising morbidity of co-infection with HIV make worse the epidemic of TB (Gandhi *et al.*, 2010).

Due to variable efficiency of BCG vaccine some researchers realized the importance to improve BCG vaccine like Grode *et al.* (2005), Soualhine *et al.* (2007), Hinchey *et al.* (2007) reported the basis for rational genetic manipulation of the current vaccine Mycobacterium bovis BCG in order to improve its protective efficacy. DNA vaccine is more efficient and cost effective especially against endoparasites like *M. tb* and *M. bovis*. To identify *M. tb* antigens as candidates for a subunit vaccine against TB, some researches like Coler *et al.* (2009), had employed a CD4+ T-cell expression screening method, demonstrated that, T-cell screening of the *M. tb* genome can be used to identify CD4+ T-cell antigens that are candidates for vaccine development. Cooper *et al.* (1993) and Jouanguy *et al.* (1996) reported critical role of IFN- γ in the control of mycobacterial infections has also been demonstrated in both mice and in humans. Recent advances in Mycobacterial genetics and genome sequencing have highlighted large number of potentially good vaccine candidates like esat6, Mpt 64, Ag85 A, B, and C, cfp10, Hspx and Hsp21 etc. Different researchers like Cole *et al*. (1998) and number of them have already been tried as DNA vaccines as reported by Grover *et al.* (2006), Jeon *et al.* (2010) and Derrick *et al.* (2004), similarly rBCG based vaccines, BCG^{+}DNA co-immunized vaccines are also tested on animal models as reported by Sugawara *et al.* (2006).

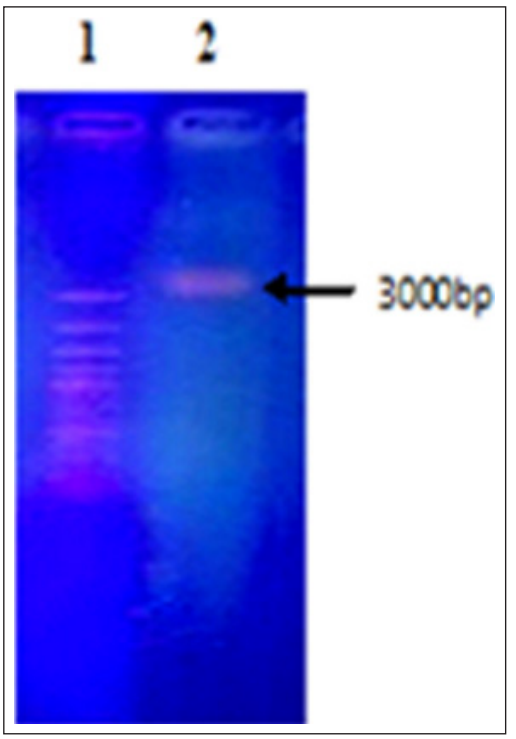

Figure 3. Confirmation of DNA. Lane 1: 100 bp ready to use DNA marker (Cat = SM0323 Thermo scientific). Lane 2: Band of extracted plasmid DNA (Rv 0378).

The current study was designed with objective to study the expression and to make clones of *M. tb* gene Rv0378. By keeping in view the importance of Rv0378 this gene was cloned and remains to be sequenced. Amplification of the gene was done to get the optimized sized DNA by using PCR technique. Primers with kozak sequence were used to see the expression of Rv0378 gene. This Kozak sequence is a well reported sequence (DNA element) to enhance expression of cloned genes inside the eukaryotic expression systems by providing additional ribosome binding sites (Kozak, 1987). Further DNA was digested with single cutter enzyme DpnI to confirm the originality of exact amplified product. Samples were ligated in order to be transformed into competent cell, single ultra-competent cell preparation kit enebled to prepare competent *E. coli* cells in a single step to transform the cells and to make clones. Hanahan *et al.* (1991) reported the widely known procedure used for preparations of ultra-competent *E. coli* by using $CaCl_2$ method. Transformation by following traditional $CaCl_2$ protocol, as different concentrations of $CaCl_2$ solution were tested to prepare and transform competent cells (Sambrook and Russell, 2001). Comparatively high yield quality of plasmid DNA (without the impurities of salts and protein) was extracted through EZ-10 Spin Column Plasmid DNA Kit. Two conformations of a DNA fragment were confirmed by loading the sample in wells with 100bp ready to use DNA marker 100bp ready to use DNA marker (Cat # SM 0323 Thermo scientific) on gel, having size of 3000bp. The extraction of DNA was also performed by Seipp *et al.* (2010) via the manufacturer's specified protocol using the Biomek® NX robot. Lu *et al.* (2011) conducted similar work as construction of recombinant plasmids pPro685A and pcD685A and also genes coding ESAT-6 (esxA, Rv3875) and Ag85A (fbpA, Rv3804c) were amplified and digested with restriction enzymes.

Plasmid DNA has been extracted for the preparation of DNA vaccine against tuberculosis by using *M. tb* gene Rv0378. Post inoculation of this vaccine will be studied in animal trial later.

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