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Cloning, Expression and Genetic Immunization Studies of *Mycobacterium tuberculosis* Gene *esat6*

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Abstract.- Early secreted antigenic target protein 6 (*esat6*) is one of the genes present on region of difference 1 (RD1) of *Mycobacterium tuberculosis* (*M. tb*) genome. This RD1 is a characteristic of virulent strains of *M. tb* and *Mycobacterium bovis* and this is one of the major differences between the disease causing strains and Bacillus-Calmat Guerin (BCG) vaccine strains. Studies have proved the presence of large number of memory T cells in *M. tb* infected individuals and these memory cells are reactive towards *Esat6* antigen, which highlighted the importance of this gene especially in early infections. In this study, numbers of *esat6* gene constructs were made in order to get a suitable construct to be used as good DNA vaccine. First *esat6* gene construct was made in pND vector without Kozak sequence upstream the gene, second construct was made in pcDNA3.1 vector with Kozak sequence upstream the gene, third construct was made again in pND vector with Kozak sequence, fourth construct was made with Kozak sequence upstream and GGG downstream the ATG as a second codon of gene first in pcDNA3.1 and later in pND vectors respectively which was designated as construct five. Sixth construct was a fusion and in pcDNA3.1 vector with Kozak upstream the gene and epitope V and poly histidine tail sequence provided by vector down stream the gene through in-frame cloning of *esat6* gene with sequences provided by vector by removing stop codon through PCR based primers. Seventh and final construct was prepared in pND14 vector also as a fusion construct and gene was cloned under tissue plasminogen activator sequence in an in-frame through PCR based primers. All these constructs were subjected to 293T human embryonic kidney cell lines to evaluate their level of expression. Although none of the constructs gave detectable level of expression in cultured cells when tested through Western blots (WB) but tpa-*esat6-pND14* construct was selected as potential DNA vaccine candidate to inject intramuscularly and interadermally to balb/c mice along with controls to obtain detectable response *in vivo*. Animals were tested nine weeks post vaccination and found positive against *tpa-esat6-pND14* vaccine through WB and multiplex micro bead immunoassay (MMIA).

Key words: DNA vaccine, esat, pND vectors, TB vaccine, balb/c mice, MMIA.

INTRODUCTION

Tuberculosis (TB) being an infectious disease can affect many parts of the body, but lungs are its preferred site to infect. Respiratory secretions such as spit or phlegm (sputum) and aerosols generated by coughing, sneezing, laughing, or breathing of *M. tb* infected patients are the common means of spread. Most of the infected individuals manage to confine few cells of *M. tb* in their body, where these cells stay alive in an inactive form. This latent TB infection does not make the patient sick or infectious and, in most cases, it does not progress to cause active tuberculosis while in other cases it may lead to pulmonary TB, which is highly contagious and fatal, if not treated appropriately. In 2010, there were 8.8 million (range, 8.5-9.2 million) incident cases of TB, 1.1 million (range 0.9-1.2 million) deaths from TB among HIV-negative people and an additional 0.35 million (range 0.32-0.39 million) deaths from HIV-associated TB (WHO, 2011).

Respiratory tract is the main route of inhalation and infection for *M. tb* bacilli, through which bacterium enter as an airborne droplet and proceeds directly to the distal lobe of lung to establish primary TB. Due to thick waxy coat and genetic potential, *M. tb* is equipped with extraordinary ability not only to persist but also to keep on dividing under hostile environments of components of mucosal and cellular immunity, where most other pathogens would not survive.

Numerous approaches to vaccine

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development are being attempted including constructing attenuated *M. tb* strains, improving the current BCG vaccine, developing subunit or DNA-based vaccines etc. The subunit vaccine approach is among the top vaccine technologies presently being evaluated, in part because subunit vaccines can meet current high safety requirements and they are amenable to standardized and reproducible production (Anderson, 2001). Comparative studies have identified 16 regions of difference (RD1-16) between the genomes of *M. tb* and BCG, of which one deletion, termed 'RD1', is absent from all BCG currently used as TB vaccines globally. RD1 is part of a 15-gene locus (ESX-1), which encodes a secretion system that enables the secretion of several proteins including *Esat6*, Cfp10 and Mpt64 which are also encoded in RD1 (Hsu *et al.*, 2003; Lewis *et al.*, 2003; Guinn *et al.*, 2004). According to Brandt *et al*. (2000) and Weinrich *et al*. (2001) the *Esat6* and Mpt64, two proteins absent in BCG have very good potential to be used as DNA vaccine. Besides *Esat6* and Mpt64, other proteins of RD regions, such as Cfp21 and Cfp10, are known to induce strong IFN-γ production, proliferation of T cells and moderate cytotoxic T cell activity in *M. tb* infected mice as reported by Weldingh *et al*. (1998). *Esat6* specific T cells are frequently found in TB patients as well as in infected animals (Brandt *et al*., 1996; Ravn *et al*., 1999; Ulrich *et al*., 1998; Raviglione *et al*., 1999). Thus, *Esat6* is being extensively studied for its potential activity as a subunit vaccine (Dietrich *et al*., 2006). Consistent with this, a recombinant BCG strain that contains region of difference 1 (RD1), which includes *Esat6*, exhibited improved protection against TB (Pym *et al*., 2003). However, the basis of this improved protection remains elusive. Furthermore, the mechanism by which *esat6* vaccination induces protective immune responses against TB remains to be investigated. By keeping in view the importance of *esat6* gene, this gene was cloned and expressed in various ways in three different eukaryotic expression vectors to choose best clone and cloning strategy for preparation of future DNA vaccine constructs. Finally, *tpa-esat6-pND* construct was evaluated for its immunization potential in mice model to standardize the immuno assays before starting vaccine challenge and preclinical studies with large number of potential DNA vaccine constructs for future studies.

MATERIALS AND METHODS

Genes and plasmids

The *esat6* gene cloned in pET 15b vector was obtained from TB Resource Center, Colorado State University, Boulder, CO, USA. Mammalian expression vectors, pcDNA3.1 Topo purchased from Invitrogen, USA and pND and pND-14 were personal gift from Dr. Gary Rhodes, UC, Davis USA. These vectors contain immediate early promoter and enhancer sequences from cytomegalovirus (CMV) for optimal gene expression in mammalian cell lines. The pND and pND14 vectors have additional 1000 bp interon region between multiple cloning sites and promoter for optimized gene expression. The pND14 vector is a modified form of pND vector; it provides additional tissue plasminogen activator sequence (tpa) upstream the cloned gene, when cloned in-frame and express product is tagged with tpa.

PCR and cloning of esat6 *gene*

The primers used for amplification of *esat6* gene are

esat6F 5′attagatctgccaccatgacagagcagcagtgg′3,
esat6R 5′aagaattctcatgcgaacatcccagtgac′3,
esat6(modified) F 5′ gagatctgccaccatggggac agagcagcagtgg′3,
esat6(modified) R 5′cgcgaacatcccagtgac′3
esat6 (modified2) F 5′ ggatccatgacagagcagcagtgg′3.

All amplifications were done by high fidelity taq polymerase and hotstart PCR technique. All PCR products were first cloned pTZRT/A vector (Fermentas, USA) or directly in pcDNA3.1 Topo and later in pNDs by using A overhangs produced by DNA polymerase and specific restriction sites available in MCS of vectors. The positive clones were confirmed by restriction digestion and sequencing analyses. Qiagen kits (Qiagen, USA) were used for DNA preparations.

Animal cell culturing

Baby hamster kidney-21 (BHK-21), HeLa

and Human embryonic kidney (293T) cell lines were propagated in tissue culture. Glutamax DMEM media from Invitrogen, USA containing 5% solution of penicillin/streptomycin and 10% fetal bovine serum (FBS) was used for culturing. The 293T cells in comparison to others were found best in transfection studies and further used in expression analysis.

Transfection studies

The complexes in different ratio of DNA: fugen6 were used for transfection studies. Before adding the complexes into the medium of cultured cells, the media was changed and serum content was reduced up to 2% in final volume. The cells with 50-70% confluence were used for transfection. The transfected cells were incubated for 72 h at 37°C with 5% CO_2 level in CO_2 incubator. The cells were harvested 72 h post transfection and expressions were confirmed by Western blotting (WB). The egfp-pND construct was used as a transfection control (Fig. 1).

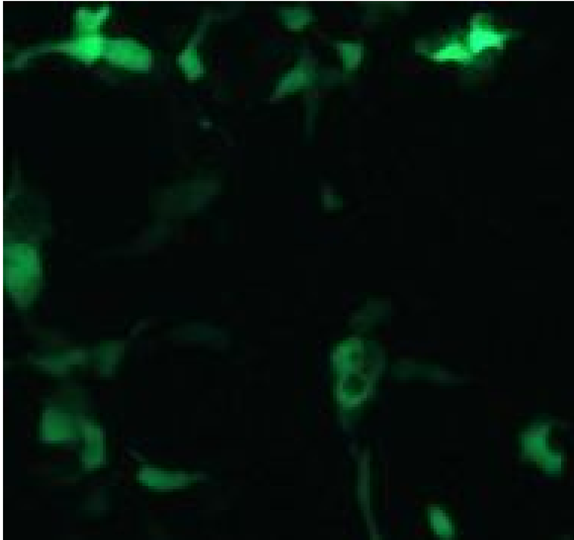

Fig. 1. The egfp-pND construct (transfection control) in 293T cells.

Sodium dodecyl sulphate (SDS)-PAGE and WB

Pre-cast 8-16% SDS-PAGE gels from Invitrogen (USA) was used to resolve the proteins from harvested cells in 1 x loading dye with 5% mercaptoethanol. The proteins were transferred on PVDF membranes by wet transfer method and standard WB procedures were used as prescribed by Bolt and Mahoney (1997). Two different detection kits *i.e*. DAB kit and ECL were used for WB.

Western blotting

WB technique was used to confirm the expression of *esat6* gene in cultured animal cell lines; similarly this technique was further used to confirm the presence of antibodies against *esat6* in *tpa-esat6-pND14* vaccinated animals.

Multiplex microbead immunoassay (MMIA)

The coupling of proteins to Luminex carboxylated microspheres was done by method described by Awan *et al*. (2012).

The MMIA, containing a mixture of six microbead sets, one for *Esat6* coated antigens along with controls like anti human IgG, biotin, cell lysate, anti mouse IgG and BSA, was used analyze the plasma samples from vaccinated and control animals, which were diluted 1:200 in Prionex (Bio-WORLD, Dublin, OH), as described by Khan *et al*. (2005). Plasma samples from negative control balb/c mice provided the baseline (background) median fluorescence intensity (MFI) for the MMIA

Animal trials

Animal protocol was approved by Institutional Animal Care and Use Committee (IACUC), UC, Davis, USA before the start of animal trial. Female balb/c mice of age seven weeks were obtained from Jackson Labs, CA (USA) and kept at animal house facility of UC, Davis USA for one week before the start of animal trials. Animals were divided into three groups. Two animals were inoculated with *tpa-esat6-pND14* plasmid and two animals were inoculated with Np-pND plasmid (containing nucleoprotein gene from H5N1 influenza virus) as positive control and two animals were used as negative control (normal saline). Animals were injected through two routes *i.e.* intramuscular (IM) and intradermal (ID). Animals were tail bled to collect the time zero blood samples and anesthetized by intraperitonial injection of ketamine before plasmid inoculations. Plasmids were inoculated at the dose of 50 µg/100 µl of normal saline/leg of animal and 25 µg/100 µl of normal saline intradermally at the base of tail. Total

125 μg DNA was delivered to each animal. Animals were bled at the every three week intervals till nine weeks and finally bled by cardiac puncture after CO_2 euthanization. The dead bodies were disposed according to the approved protocol.

Post vaccination antibody profiling

Post vaccination sera were collected in serum separating gel tubes (BD Diagnostics, USA). The antibody response against *Esat6* was detected by WB and MMIA.

RESULTS

esat6-pcDNA3.1 *Topo clone*

The Topo construct of esat6 was confirmed by BamHI and XhoI enzymes and 365 bp fragment was produced as shown in Figure 2.

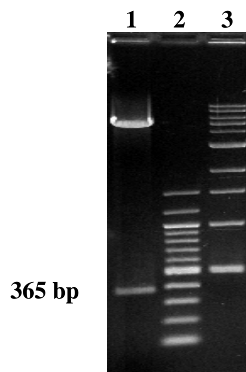

Fig. 2. Restriction digestion of *esat6-pcDNA3.1* Topo clones (construct 2). Lane 1. *esat6-pcDNA3.1* Topo clone with BamHI and XhoI. Lane 2. 100 bp DNA marker (Cat # N3231L, New England Biolabs, USA). Lane 3. 1kb DNA marker (Cat # N 3232S, New England Biolabs, USA).

Expression studies

The results of expression of *esat6* clones are shown in Figure 3. Figure 3A shows the results of clones having construct 1 (*esat6*-pND without Kozak sequence clones). The band appeared in positive control (lane 1) but the same was missing in next two lanes; *i.e.* 2 and 3. Lane 2 represents the lysate of 293T cells (untransfected cells) and lane 3 represents the lysate of transfected cells with *esat6*-pND construct (without Kozak sequence). In next experiment, the expression of clones containing construct 2 *i.e. esat6-pcDNA3.1* with Kozak sequence and construct 3 *i.e. esat6*-pND with Kozak sequence were checked but no detectable expressions were observed in WBs. Similarly, no detectable expression of clones with construct 4 (*esat6-pcDNA3.1* with Kozak sequence upstream the gene and GGG codon downstream the ATG) was observed. Figure 3B represents the expression study of construct 5 (*esat6-pND* having Kozak sequence upstream the gene and GGG codon downstream the ATG). Even addition of this extra G in the form of GGG codon in order to complete the Kozak sequence did not help to give detectable expression (Fig 3B, Lane 4-8). Later the clones with two fusion constructs of *esat6* genes were tested in animal cell lines separately but results from both experiments were found to be negative as clear in Figure 3C which represents the results of expression studies of clones having construct six with *esat6-pcDNA3.1* + Kozak upstream and epitope V + poly His tail sequence downstream the gene. In case of clones having *tpa-esat6-pND14* construct, the expressed *Esat6* protein was not only checked in transfected cells but also in culture media. But detectable amount of expressed protein could not be found in both or either. The polyclonal anti *Esat6* antiserum was used in each WB experiment except for clones with constructs five and six, for which monoclonal anti His antibody was used. While for clones with construct seven monoclonal anti *Esat6* antiserum was used along with polyclonal anti *Esat6* antiserum.

Animal trial studies

Finally to check whether or not immune responses generated in vaccinated animals. The antiserum from vaccinated animals were collected and tested by WB and MMIA. The results of WB showed the presence of specific antibodies against *Esat6* (Fig. 4). Negative and positive serum controls were also used to make sure that obtained results are not art fact.

In case of MMIA, antiserum was not only tested against *Esat6* antigen but also against anti-human IgG, biotin, cell lysate, anti-mouse IgG and BSA antigens which worked as internal controls of MMIA. The median fluorescence intensity (MFI) from *tpa-esat6-pND14* vaccinated animals was

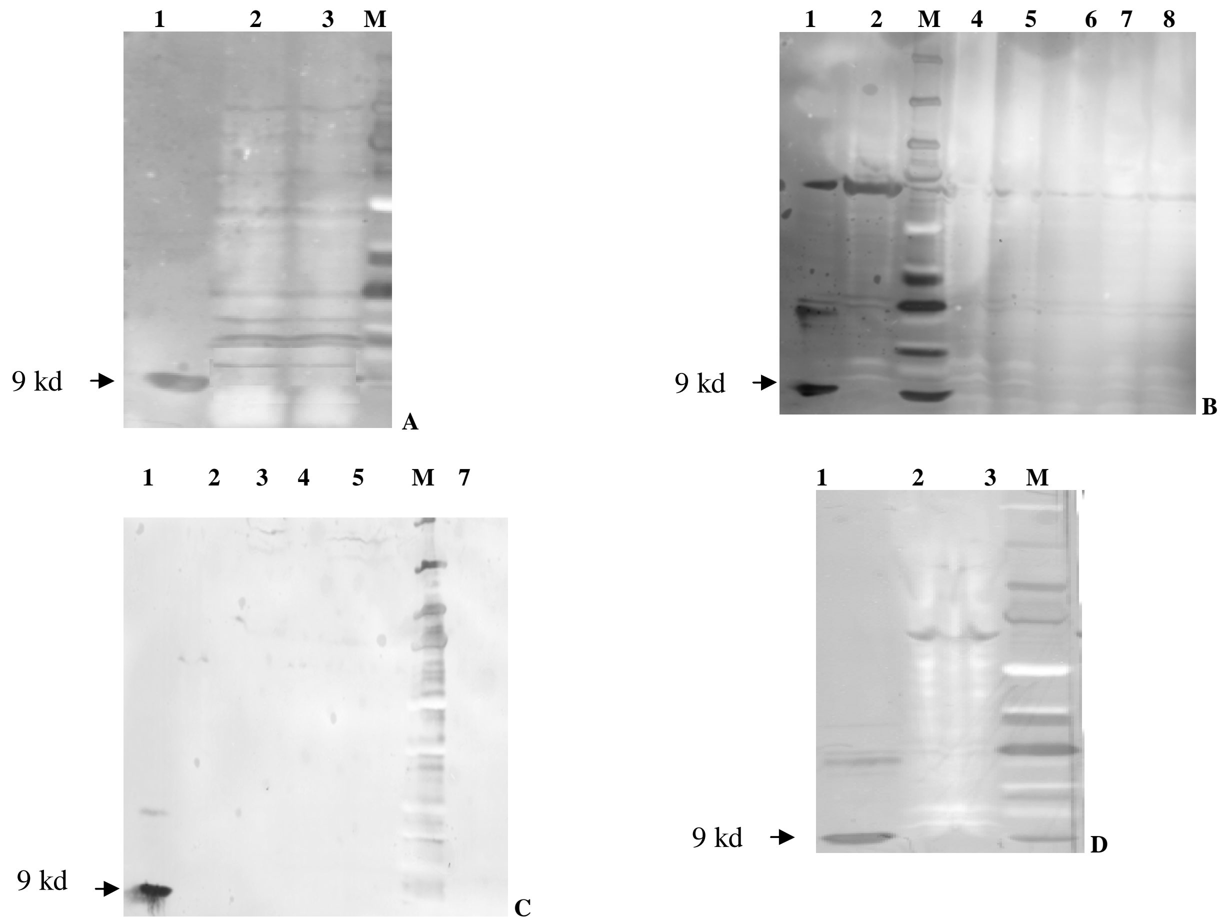

Fig. 3. Western blot showing the results of expression studies of various esat6-pND clones. **A)** Clones without Kozak sequence with polypeptide specific antiserum. Lane 1: Affinity based column purified Esat6 protein as positive control. Lane 2: 293 T cells (untransfected). Lane 3: 293T cells transfected with esat6-pND clone without Kozak sequence. Lane 4: Precision plus MW marker (Bio Rad, USA). **B)** Clones with Kozak sequence and additional GGG codon downstream ATG of esat6 gene with polypeptide specific antiserum. Lane 1: Affinity based column purified esat6 protein as positive control. Lane 2: 293T cells (untransfected). Lane 3: Precision plus MW marker (Bio Rad, USA). Lane 4-8: 293T cells transfected with esat6-pND with additional GGG in sequence after ATG. **C)** Clones of esat6-his-pcDNA3.1 Topo with anti His antibody. Lane 1: Affinity based column purified esat6 protein as a positive control. Lane 2-5: The 293T cells transfected with esat6-his-pcDNA3.1 Topo. Lane 6: Precision plus MW marker (Bio Rad, USA). Lane 7: 293T cells (untransfected). **D)** Clones of tpa-esat6-pND14 with polypeptide specific antiserum. Lane 1: Affinity based column purified esat6 protein. Lane 2: The 293 T cells transfected with tpa-esat6-pND14 clone. Lane 3: The 293 T cells untransfected Lane 4: Precision plus MW marker (Bio Rad, USA).

found to high in comparison to negative and day zero control animals. The results of MMIA are shown in Table I and MFI from vaccinated animals is highlighted in blue. The MFI against esat6 (bead 177) and IgG (bead 186) was higher in both vaccinated animals as compared to negative controls, which confirmed the production of anti Esat6 immunoglobulin in vaccinated animals. High MFI values against anti mouse IgG, anti human IgG and biotin represents the accuracy of these internal positive controls, similarly low MFI values against animal cell lysates and BSA antigens represents the low level of cross reactivity from antiserum of vaccinated animals and both of these parameters act as internal negative controls of MMIA test.

Table I.- MMIA results of tpa-esat6-pND14 vaccinated and control groups. All samples were run in duplicate and average values are noted below 1:2 dilutions are used for positive and negative control sera. Esat6 antigen is coated on bead type 177 and rest of each bead set is coated with special antigen like anti human at 106, biotin at 135, animal cell lysate at 159, anti mouse IgG at 186 and BSA at 188 are coated on different beads. All of these specifically coated antigen beads sets are used as internal controls of MMIA test.

Sample	106 Anti human	135 Biotin	159 Cell Lysate	177 Esat6	186 Anti mouse IgG	188 BSA
Blank	2249	21373	48	11	65	23
-ve 1	11601	19725	46	54	1120	35
-ve 2	13646	20552	50	143	1126	49
mice 1 (1:100)	12089	21066	32	189	1230	51
(1:200)	14013	20400	54	201	1073	133
mice 2 (1:100)	12404	20651	45	160	1178	82
(1:200)	12787	20993	72	135	1152	41
+ve (1:200)	12007	20910	53	7233	1179	54

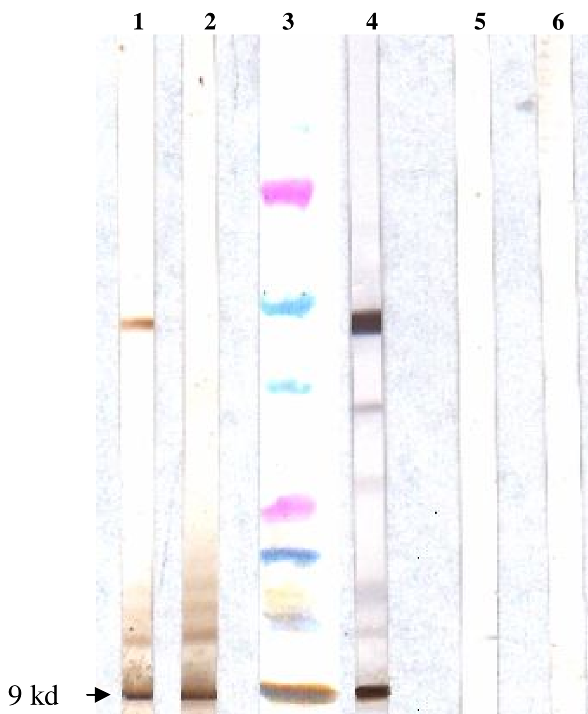

Fig. 4. Western blot showing the results of tpa-esat6-pND14 vaccinated animals. Lane 1-2: antiserum from tpa-esat6-pND14 vaccinated animals. Lane 3: Precision plus MW marker (Bio Rad, USA). Lane 4: Esat6 protein probed with known positive serum. Lane 5-6. Esat6 protein probed with negative serum.

DISCUSSION

TB is a pre-historic disease; caused by *Mycobacterium* spp. and it significantly infects large number of human and animal populations each year as reported by Sterling *et al*. (2000). To control TB is not only difficult due to non-availability of good vaccines but also because of emergence of increasingly more resistant strains like multiple drug resistant (MDR) and extremely drug resistant (XDR) (Smith, 2007). The non-availability of short term treatments and lack of good vaccines are another reason to control TB. DNA vaccine a new and state of the art strategy is more efficacious and cost effective especially against endoparasites like *M. tb* and *M. bovis*. 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. (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) and Romano *et al*. (2006). Almost all of these rBCG vaccines have successfully immunized and later protected the animals upon challenge. The new BCG vaccines are required because the existing BCG vaccine is almost a hundred years old and it is now one of the most controversial vaccines in the world which provides protection only till the age of five among vaccinated individuals (Baily, 1980). The esat6 gene located on RD1 region of *M. tb* genome is under extensive investigation since last ten years and according to Behr *et al*. (1999), this

molecule is involved in virulence of *Mycobacterium* spp. According to number of researchers around the world the *in vitro* expression and *in vivo* humoral response of native esat6 gene is low as tested through animal cell lines and mouse models. This gene is cloned with modifications to enhance its cell line based expression, which in return increase its humoral response *in vivo* (Wang *et al*., 2004, 2009; Shi *et al*., 2005; Oveissi *et al*., 2010). The current study was designed with objective to study expression and later humoral response of *esat6* gene under eukaryotic expression systems by using different upstream modifications and even three different well known eukaryotic expression vectors pcDNA3.1, pND and pND14 were used. The modifications were introduced through PCR based primers and 1-7 constructs were made by using these primers. Primers without Kozak sequence were used to see the expression of *esat6* gene cloned into pND by using the natural sequence of gene under constitutive CMV promoter of vector. No results on WB by this clone indicated that these natural conditions are not good enough to get adequate level of expression of *esat6* gene. Later the constructs 2 and 3 were designed with well reported Kozak sequence but again no results were seen through these esat6-pcDNA3.1 and esat6-pND constructs respectively. 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). Another primer based modification was introduced and this time Kozak sequence is not only introduced upstream the gene but for the of sake of addition of G after ATG, GGG codon was introduce and upon addition of this codon, glycine residue was additionally introduced after formyl-methionine on N-terminal of protein. This modification was introduced to provide complete Kozak sequence *i.e.* GCCACCATGG as an attempt to get expression of this gene. The constructs were made in pcDNA3.1 and pND vectors respectively as a construct 4 and 5. After failure of getting expression through these constructs, the esat6 gene was cloned as a fused or conjugated molecule with immune dominant tags like tpa, epitope V and poly Histidine. First the esat6-pcDNA3.1-epitope V-His construct was made and tested for expression by using anti His monoclonal antibody and later tpa-esat6-pND14 construct was made by in-frame fusion of esat6 with tpa provided by pND14 vector upstream the gene. Very poor levels of expressions were seen through these constructs on WBs. The expression from all the constructs was confirmed by Esat6 polypeptide specific polyclonal antiserum as well as by Esat6 polypeptide specific monoclonal antisera provided by TB resource center, Colorado State University, Colorado, USA. Although number of dilutions of Esat6 specific antiserum was used but no convincing results were seen through any WB. Despite having no expression of esat6 gene under *in vitro* conditions tpa-esat6-pND14 construct was selected to be used as DNA vaccines because tpa conjugated vaccines are generally more potent than native one as reported by Casimiro *et al*. (2002). Only two animals were used for vaccine group along with two animals in each control group because this is a confirmatory study and immune response against esat6 gene is already reported through number of studies (Fan *et al*., 2007; Sang *et al*., 2012). Further this is a pilot study to set the plate form for development and testing of more and more and unique genes from *M. tb* and *M. bovis* as a DNA vaccine to control human and bovine TB. The tpa-esat6-pND14 construct provided good humoral response in mice model as confirmed by MMIA and WB. MMIA is one of the most recent immunoassays, its principle is based on ELISA and it is most convenient, cost effective and robust diagnostic technique (Khan *et al*., 2006). This technique was indigenously developed in collaboration with University of California, Davis, USA and Pir Mehr Ali Shah Arid Agriculture University Rawalpindi, Pakistan (Awan *et al*., 2012). The pND vectors used in this study are one of the successful vectors to be used as DNA vaccines as reported by Lena *et al*. (2002). The gene for enhanced green flouriscent protein (egfp) was cloned into pND and later use as transfection control (Fig. 1), the expression of this gene confirm the efficiency of plasmid and its promoter. The presence of anti Esat6 antibodies in vaccinated animals confirms the antigenic potential of esat6 gene as it is delivered through DNA vaccines but the cell mediated response and vaccine challenge studies are

need to be done.

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