Reports of Biochemistry & Molecular Biology
Vol. 2, No.1, Oct 2013

Original paper

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Construction of eukaryotic expression vectors encoding CFP-10 and ESAT-6 genes and their potential in lymphocyte proliferation

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Abstract

Background: *Mycobacterium (M.) bovis* is the agent of bovine tuberculosis (TB) in a range of animal species, including humans. Recent advances in immunology and the molecular biology of *Mycobacterium* have allowed identification of a large number of antigens with the potential for the development of a new TB vaccine. The ESAT-6 and CFP-10 proteins of *M. bovis* are important structural and functional proteins known to be important immunogens.

Methods: In the current study, the DNAs encoding these genes were utilized in the construction of pcDNA 3.1+/ESAT-6 and pcDNA3.1+/CFP-10 plasmids. After intramuscular injection of BALB/c mice with these plasmids, ESAT-6 and CFP-10 mRNA expression was assessed by RT-PCR. Mice were inoculated and boosted with the plasmids to evaluate their effects on lymphocyte proliferation.

Results: Our results indicate the plasmids are expressed at the RNA level and can induce lymphocyte proliferation.

Conclusion: Further study is needed to characterize the effect of these antigens on the immune system and determine whether they are effective vaccine candidates against *M. bovis*.

Keywords: *Mycobacterium bovis*, DNA vaccine, ESAT-6, CFP-10, PPD, Proliferation assay, BALB/c mice

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Introduction

Mycobacterium (M.) bovis is a slow-growing, aerobic bacterium and the causative agent of tuberculosis (TB) in many domesticated and wild animals, in particular *Bovidae*, *Cervidae*, and occasionally carnivores and humans (1-3). More than 10% of human TB is caused by *M. bovis*. Bovine TB is a zoonotic disease that is seriously detrimental to the dairy industry (4). If a country's animals are infected with bovine TB, humans will always be threatened by it. If action is not taken to eliminate *M. bovis*, the control of human TB will be unsuccessful (5). The disease can occur sporadically and is difficult to eradicate. The introduction of milk pasteurization in developed countries has dramatically reduced the transmission of TB from cattle to humans (6) but the causative agent of bovine TB, *M. bovis*, remains responsible for 5-10% of human TB cases (7). Artificial immunization and natural infections are difficult to differentiate in cattle and other animals, because after vaccination with Bacillus Calmette–Guérin (BCG), the results of allergic detection tests are always positive. Skin tests and slaughter policies have significantly reduced the incidence of bovine TB; however, trials have shown that the attenuated BCG vaccine does not provide consistent protection against TB (8, 9). Several major antigens such as

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Received: Jul 12, 2013; Accepted: Aug 10, 2013

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hsp60, Ag85, ESAT-6, and others have shown promise as new vaccine candidates (10, 11). Using these antigens, a number of diagnostic systems and vaccines have been developed (12).

The 10-kDa culture filtrate protein (CFP-10) and 6-kDa early-secreted target antigen (ESAT-6) are two low molecular weight secretory proteins encoded by the Rv3874 and Rv3875 genes, respectively. Both genes are located in the region of difference-1 (RD1) region of the *Mycobacterium (M.) tuberculosis* (MTB) genome, but absent in all *M. bovis* BCG vaccine strains. These proteins form a 1:1 heterodimeric complex, known as the CE protein (13). The ESAT-6/CFP-10 complex's secretion from and attachment to infected host cells demonstrates its contribution to the pathogenicity of *M. tuberculosis* (14).

The presence of antibodies against ESAT-6 and CFP-10 in patients with TB was confirmed, and it was demonstrated that significant antibody responses are not restricted to active TB disease but can also reflect latent infections (17).

Analysis of the MTB immunoproteome using high-throughput detection of antibodies in human serum against the entire MTB proteome revealed that CFP-10 is significantly associated with active TB (18).

ESAT-6 has recently been demonstrated to induce protective immunity when administered as a subunit or DNA vaccine (19).

Roles for these molecules in future TB vaccines have previously been suggested and these antigens have shown promise when delivered as DNA vaccines (20-23). The purpose of the present work was to construct recombinant plasmids encoding the ESAT-6 and CFP-10 antigens as DNA vaccines, characterize their expression in BALB/c mice, and inoculate and boost mice to evaluate the plasmids' effects on lymphocyte proliferation.

Materials and Methods

Bacterial Strains, Plasmid and mice

Purified DNA of *M. bovis* (strain NA5) and bovine PPD (PPD-B) prepared from *M. bovis* were obtained from the PPD Tuberculin Department, Razi Vaccine and Serum Research Institute, Karaj, Iran (RVSRI).

Escherichia (E) coli strain Top 10 (Stratagene, USA) was used for cloning. The bacteria were grown in or on Luria-Bertani medium (LB) broth or agar supplemented, when required, with 50 µg/ml of ampicillin.

The eukaryotic expression vector pcDNA3.1(+) (Novagen, USA) was used for plasmid construction. All chemicals were purchased from Merck & Company, Inc. (Germany). Female BALB/c mice, 6-8 weeks of age, were provided by the animal house of RVSRI.

PCR amplification and cloning of ESAT-6 and CFP-10

ESAT-6 and CFP-10 genes were amplified by PCR from the purified *M. bovis* strain NA5 DNA by the specific primers designed using Gene Runner and BioEdit software and data in Genbank, NCBI. The restriction sites were designed into the primers. The primers are shown in Table 1.

Table 1. Primers used in this study. Enzyme restriction sites are underlined.

Gene	Sequence		Restriction site
ESAT-6	Forward	AGCA/AGCTTATTATGGGAACAGAGCAGCAGTGGAA	*Hind* III
	Reverse	TATGCGAACATCCCAGTGACTGAGAGCT/CGGTTAAAGC	*Xho I*
CFP-10	Forward	TAGA/AGCTTGATATGGGAGCAGAGATGAAGACCGATG	*Hind III*
	Reverse	GAAGCCCATTTGCGAGGATGACGCCGG/CGCTA	*Not I*

PCRs were performed in a 25 µl volumes containing 100 ng of template DNA, 0.5 µl (0.5) µM of each primer, set-specific for ESAT-6 or CFP-10, 2.5 mM MgCl2, 200 µl (2.5 mM) each) deoxynucleoside triphosphates (dNTP), 1X PCR buffer, and 1.5 unit of *Pfu* DNA polymerase. The following condition was used for the amplification: hot start at 94 ^{o}C for 5 min, followed by 35 cycles of denaturation at 94 ^{o}C for 1 min; annealing at 58 ^{o}C for 45 s; and extension at 72 ^{o}C for 1 min. The PCR products were analyzed by electrophoresis on a 1.5 % agarose gel in TAE buffer and visualized by SYBR Green staining on a UV gel documentation system.

The PCR products were purified from a 1.5% low-melting agarose gel with a "PCR Product Extraction Kit" (Fermentas) according to the

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manufacturer's instruction. The purity of the gel-extracted PCR product was determined on a NanoDrop ND-100 spectrophotometer.

Purified PCR products were subjected to two-step digestion with *Xho* I/*Hind* III and *Not* I/*Xho* I restriction enzymes for ESAT-6 and CFP-10, respectively. The purified, digested PCR products were ligated into pcDNA3.1(+). Standard techniques for these steps such as plasmid DNA preparation, ligation, competent cell preparation, and transformation were followed as described previously (24). Competent *E. coli* TOP10 cells (Invitrogen, Carlsbad, CA) were transformed with pcDNA3.1+/ESAT-6 and pcDNA3.1+/CFP-10 using a transformation kit (Fermantas, Lithuania). Ampicillin-resistant cultures were grown in LB medium containing 50 µl/ml of ampicillin at 37 ^{o}C with shaking until OD at 600 nm = ~ 0.5. The fidelity of the transformants was verified by PCR using ESAT-6 and CFP-10-specific primers that had previously been used for amplification. The plasmids were purified and restriction enzyme-digested. After electrophoresis on a 1.5% agarose gel, the purified plasmids were sequenced (Seq Lab, microgen).

To assess expression, four mice received 100 µg of pcDNA3.1+/ESAT-6 or pcDNA3.1+/CFP-10 recombinant plasmids by intramuscular injection. One mouse received sterile PBS and one mouse was not injected (CNT-). Total RNA was isolated from the muscle 72 hours post-inoculation using a TriPure kit (Roche). The samples were analyzed by RT-PCR with specific ESAT-6 or CFP-10 primers, GAPDH primers as an internal control and a negative control.

Inoculation of mice with plasmids encoding ESAT-6 and CFP-10

Plasmids were purified from transformed *E. coli* Top 10 cells by the alkaline lysis method, resuspended in sterile PBS pH 7.2, and stored at -20 ^{o}C. The plasmid concentrations were measured by NanoDrop at 260 nm. Mice received injections of 100 µg of DNA resuspended in 100 µl of PBS into the right quadriceps muscle using an insulin syringe. Table 2 describes the delivery program and mouse groups.

Forty-eight mice were separated into six groups of eight mice each, and all eight in each of five groups were injected with the following: pcDNA3.1+/ESAT-6, pcDNA3.1+/CFP-10, pcDNA3.1+/CFP-10 plus pcDNA3.1+/CFP-10, pcDNA3.1(+), and PPD-B. One group was kept as a control without injection (CNT-). Thirty days post-injection, the mice in each group were separated into two groups, one for a second injection, and the other for proliferation assays with spleen lymphocytes. Sixty days post-injection spleens were harvested from all remaining mice for lymphocyte proliferation assays.

Spleen lymphocyte proliferation assay (MTT assay)

Mouse spleen cells were isolated by homogenizing the organ in RPMI 1640 medium, supplemented with penicillin (100 IU/ml), streptomycin (50 µg/ml), and 10% fetal calf serum (FCS). To isolate mononuclear cells, 5-ml aliquots of the spleen cell suspension were layered onto 2.5 ml of Ficoll (Sigma) and centrifuged at 1,000 × g for 15 min at room temperature. The cells were then gently removed from the medium-Ficoll interface and washed with RPMI medium. The isolated lymphocytes were resuspended in 5 ml of RPMI complete medium and counted on a hemocytometer.

The lymphocytes (5×10^4/well) were plated in duplicate in 96-well tissue culture plates and stimulated with 25 µg of PPD-B for 72 h in 5% CO_2, 95% humidified air. In each plate, at least 4 wells were considered as negative controls, without adding PPD-B. Concanavalin A (Con A), 5 µg/ml, was added as a positive control in 4 wells to determine the proliferative response of cultured lymphocytes. Then, 10 µl of MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; Sigma) (5 mg/ml) were added to each well, plates were incubated at 37 °C for 4 h, and 100 µl of isopropanol with 0.04 N HCl was added and mixed thoroughly by repeated pipetting for color development. After 30 minutes the absorbance was measured using an enzyme-linked immunosorbent assay (ELISA) plate reader at a wavelength of 570 nm.

Statistical analysis

Prism (Version 4, GraphPad Software, Inc.) was used for statistical analyses. The statistical significance of the difference between groups was determined by the one-way ANOVA test. $P < 0.05$ was considered significant.

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Results

Cloning

The genes encoding ESAT-6 and CFP-10 from *M. bovis* AN_5 were separately cloned into the pcDNA3.1+ expression vector. The integrity of the constructed vectors pcDNA3.1+/ESAT-6 and pcDNA3.1+/CFP-10 was confirmed by PCR (Fig. 1), restriction digestion analysis, and sequencing.

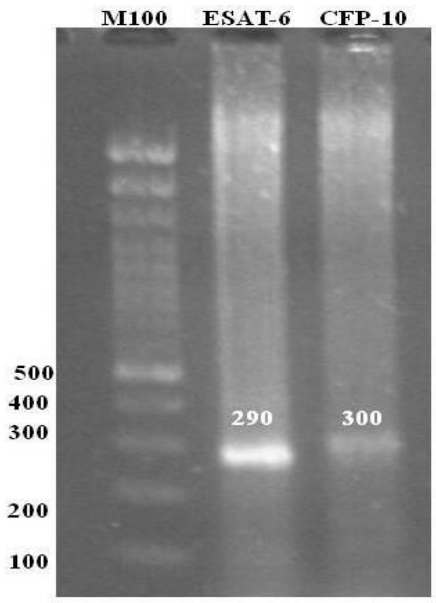

Fig. 1. Agarose gel electrophoresis of ESAT-6 (290 bp) and CFP-10 (300 bp) PCR products, pcDNA3.1+/ESAT-6, and pcDNA3.1+/CFP-10 were used as templates for amplification. Lane M100: 100 bp DNA ladder (Fermentas).

Proliferation assays

The means of the optical densities (OD) with standard deviations (SD) for mice groups are shown in Table 3. Optical density is an index for lymphocyte proliferation.

The results of the MMT assays after the first plasmid injections (I) are shown in Fig. 3. Lymphocytes of mice immunized with pcDNA3.1+/ESAT-6, and/or pcDNA3.1+/CFP-10, and PPD-B, were stimulated significantly more than those of the control groups that received PBS, pcDNA3.1(+), or no injection. This proliferation was significantly greater in the group that received both recombinant plasmids than either group that received either recombinant plasmid alone. Proliferation was greater in all treated groups than control groups. Proliferation was also greater in the group that received both plasmids than the groups that received either plasmid individually. Results were analyzed using *Bonferroni's Multiple Comparison Test*.

The statistical analyses of the MTT assays from the first and second injections showed the only group that was found significantly different between injections I and II was the ES+CF group.

To confirm that the constructs are functional in mammalian cells, they were injected into mouse muscle, and mRNA expression was evaluated by RT-PCR. The presence of the expected amplicons following RT-PCR indicated that the plasmids were transcribed in the myocytes (Fig. 2).

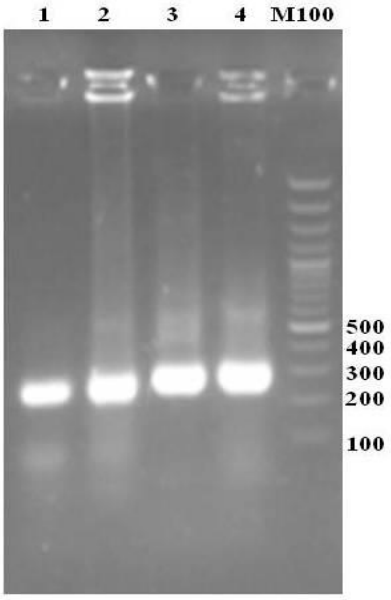

Fig. 2. Agarose gel electrophoresis of ESAT-6 and CFP-10 RT-PCR products. Lanes 1 and 2: ESAT-6 (290 bp), lanes 3 and 4: CFP-10 (300 bp), lane M100: 100-bp DNA ladder (Fermentas). In negative control was not found any amplification.

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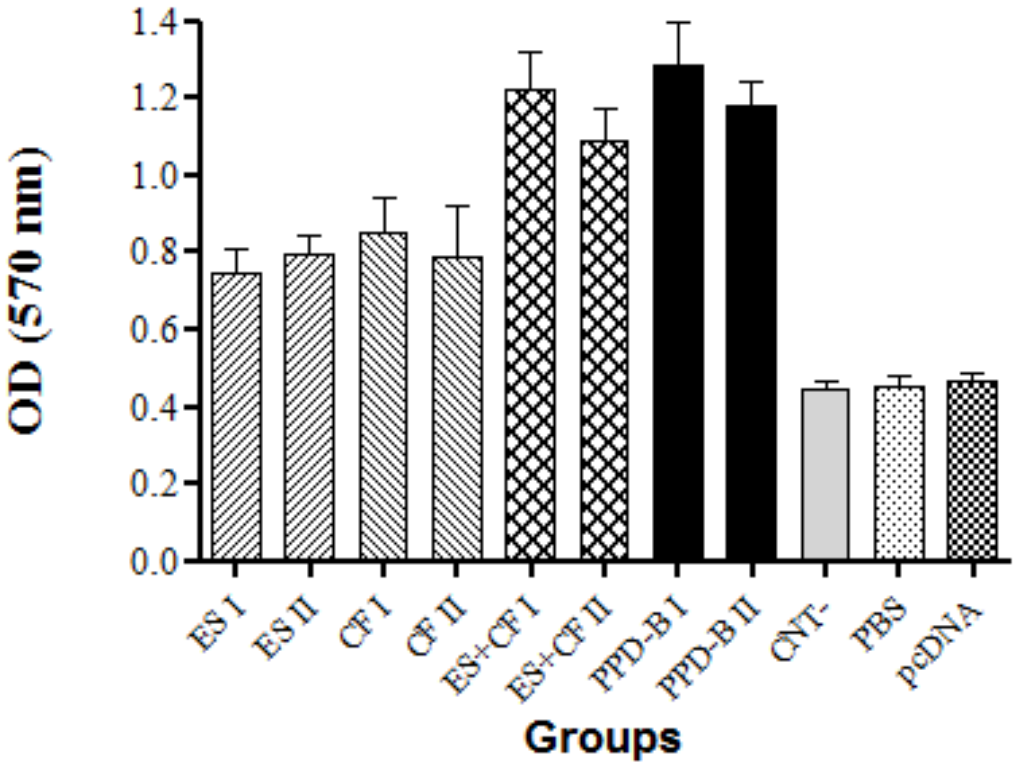

Fig. 3. The comparison of MMT assay results in mice groups in two inoculations (first: I & second: II). The proliferation has been decreased significantly in ES+CF group after booster. Groups which received ES: pcDNA3.1+/ESAT-6, CF: pcDNA3.1+/CFP-10, ES+CF: pcDNA3.1+/ESAT-6 +pcDNA3.1+/CFP-10, pcDNA: pcDNA3.1+, PPD-B: PPD-B, are shown horizontal axis.

Table 2. Mice groups used in the study.

Explanations	Name	I/ 0	II/ 20	III/ 40
pcDNA3.1+/ESAT-6	ES	12	4	P
			P	-
pcDNA3.1+/CFP-10	CF	12	4	P
			P	-
pcDNA3.1+/ESAT-6 + pcDNA3.1+/CFP-10	ES+CF	12	4	P
			P	-
pcDNA3.1+	pcDNA	8	4	P
			P	-
PPD- Bovine	PPD-B	8	4	P
			P	-
Control (no injection)	CNT-	8	4	P
			P	-
PBS	PBS	8	4	P
			P	-

I: Day 0, First injection, II: Day 20, Second injection or culture of lymphocytes of spleen and proliferation assay (P), III: Day 40, Culture of lymphocytes of spleen and proliferation. assay (P), *In each cell, the number of mice per group is shown.

Table 3. Optical densities from MTT assays.

	OD (Mean± SD)	
	I	**II**
ES	0.7444±0.1294	0.7970±0.0868
ES+CF	1.2205±0.1980	1.0874±0.1689
CF	0.8496±0.1769	0.7903±0.2617
CNT-	0.4156±0.02397	0.4475±0.04175
PBS	0.4012±0.04330	0.4493±0.05730
pcDNA	0.4283±0.04898	0.4668±0.04126
PPD-B	1.2824±0.2325	1.1778±0.1345

I: Day 0, First injection,
II: Day 20, Second injection or culture of lymphocytes of spleen and proliferation assay (P).

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Discussion

Recently, awareness of the importance of bovine TB has increased, with the emphasis on detection, diagnosis, and management, but the infection has not been eliminated.

Effective resistance to mycobacterial infections is mediated by both innate and adaptive mechanisms of immunity (25). Recognition of mycobacterial antigens by T cells generates cell-mediated immunity (26, 27). Macrophages present antigens via both class I and class II major histocompatibility complex (MHC) molecules, resulting in effector CD4 and CD8 T cells that secrete cytokines, including gamma interferon (IFN-γ), and give rise to macrophage activation and intracellular killing of the organism (27, 28).

The identification of strong antibody responses against CFP-10 and ESAT-6 proteins resulted in their wide use for the serodiagnostic detection of TB infections (30-32). Application of these proteins as vaccine candidates is controversial. This controversy may be caused by the lack of potency of these antigens to stimulate cell-mediated immune responses.

We demonstrated lymphocyte proliferation in mice that were injected with the recombinant plasmids, indicating that ESAT-6 and CFP-10 antigens were expressed. We detected no significant difference in proliferation potency between the two greater recombinant plasmids, but proliferation was in the mice co-immunized with both plasmids than those immunized with either plasmid alone. Lymphocyte proliferation was significantly higher after the first injection than the second in the co-immunized mice.

Proliferation assays using lymphocytes from PBS- or pcDNA3.1-injected mice were not significantly different that those of uninjected mice (Fig. 3).

In group that received PPD-B, we found a non-significant increase in lymphocyte proliferation compared with control groups. Because PPD-B includes ESAT-6 and CFP-10, it is possible that modulatory effects of other proteins present in PPD-B may suppress the proliferative effects of ESAT-6 and CFP-10 seen in our study.

Further studies to more completely characterize the effects of our construct, have been proposed. Identification of the cytokines produced is essential to see which types of T cells; TH1 and/or TH2, are evoked following immunizations using different treatments and delivery routes.

Acknowledgements

This study is founded by Razi Vaccine & Serum Research Institute (RVSRI) and hereby we express our thanks to the supportive personnel of Razi Institute in Mashhad, AliReza Adeli, Shahrokh Sarmadi and Kazem Boozari.

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