Immune responses against *Mycobacterium tuberculosis*-specific proteins PE35 and CFP10 in mice immunized with recombinant *Mycobacterium vaccae*

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ABSTRACT

الأهداف: لاستنساخ والتعبير عن المتفطرة السل والبروتينات PE35والبروتين الراشح الثقافي (CEP)في 10المتفطرة (M Vaccae). وفيما بعد، تقيم استجابة المناعة الخلطية والخلوية ضد هذه التركيبات المؤتلفة في الفئران.

الطريقة: من أجل ذلك قمنا باستنساخ الحمض النووي المناظر للجينات pe35 و cfp10 إلى البلازميد المكوكيpDE22؛ ومن ثم أدخلنا البلازميدات المؤتلفة الناتجة (pDE22–pe35 و pDE22–cfp10) إلى M.vaccae. بعد ذلك تم اختبار قدرة هذه المؤتلفات على تحفيز إنتاج البروتينات بواسطة Western immunoblotting مع استخدام الأمصال التي تحتوي على الأجسام المضادة PE35 و CFP10 والمنتجة في الأرنب. علاوة على ذلك، إستخرجنا أمصال وخلايا الطحال من 5 فئران التي كان قد تم حقنها بالبلازميدات المؤتلفة السابقة وذلك لاختبار الاستجابات الخلوية والخلطية وفحوصات الأجسام المضادة. وأجريت التجارب في المختبر لدينا، كلية الطب، جامعة الكويت، الكويت في الفترة بين 2009م و 2011م.

النتائج: أكدت نتائج تجارب إيمونوبلوتس الغربية (Western immunoblotting) تفعيل إنتاج البروتين PE35 من البلازميد المركب pDE22–pe35. ولكننا لم نستطع اثبات ذلك بالنسبة للبروتين CFP10. ومع ذلك، أظهرت التجارب مع الخلايا الطحالية من الفئران وجود استجابة مناعية خلوية رداً على التحصين بـ–rVaccae pDE22–pe35 وpDE22–cfp10– rVaccae. مما يؤكد إنتاج البروتينين PE35 و CFP10 من خلال البلازميدين المؤتليفين اللذين تم تركيبهما في المختبر داخل أجسام الفئران. بالإضافة إلى ذلك،فإن اختبار الأمصال أظهرت إيجابية تفاعلية مع مجموعة الببتيدات المتداخلة والتي تغطي البروتين PE35 وببتيد واحد، وهو P2، في الفئران المحصنة ب rVaccae–pDE22–pe35.

خاتمة: توصلنا إلى أن البلازميدين المؤتليفين PE35– و CFP10– واللذين تم ادخالهما في M.vaccae، هما صالحين لدراسة دور البروتينين CFP10وPE35 في الاستجابة المناعية ضد بكتيريا السل M.tuberculosis داخل أجسام حيوانات الفئران المخبرية.

Objectives: To clone and express *Mycobacterium tuberculosis* (*M. tuberculosis*) proteins PE35 and culture filtrate protein (CFP)10 in *Mycobacterium vaccae* (*M. vaccae*), and subsequently, evaluate the humoral and cellular immunity responses against these recombinant constructs in mice.

Methods: The DNA of PE35 and CFP 10 genes were cloned into the shuttle plasmid pDE22, and the recombinant plasmids were electroporated into *M. vaccae.* The recombinant constructs were then tested for expression of PE35 and CFP10 by Western immunoblotting using rabbit anti-sera. Furthermore, splenocytes and sera from groups of 5 mice immunized with recombinant *M. vaccae (*rVaccae) were tested for cellular and humoral responses in proliferation, and antibody assays. Experiments were carried out in the laboratory of the Faculty of Medicine, Kuwait University, Safat, Kuwait between 2009 and 2011.

Results: The results of Western immunoblot suggested the expression of only PE35. However, splenocyte assays showed positive proliferation in response to peptide pools, and 4 and 5 of the 6 overlapping synthetic peptides covering the sequence of PE35 and CFP10. In addition, positive antibody reactivity was detected with PE35 peptide pool and a single peptide, namely, P2.

Conclusion: The expression of PE35 and CFP10 proteins in rVaccae constructs led to the induction of cellular immune responses to multiple epitopes.

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Tuberculosis (TB) is a global health concern and a major cause of illness and death worldwide, especially in low and middle-income countries with a high incidence of human immunodeficiency virus infection/acquired immunodeficiency syndrome (HIV/AIDS) co-infection.[1] It is estimated that one-third of the world's population is infected with *Mycobacterium tuberculosis* (*M. tuberculosis*), leading to approximately 9.4 million active cases, and 1.7 million deaths each year.[2] The worldwide problem of TB is due to several factors, such as poverty, overcrowding, breakdown of national infrastructures, immigration of people from high prevalence nations to industrialized countries, and the aging of the world population.[3] The situation is also complicated by a worldwide increase in cases caused by multi-drug resistant and extensively-drug resistant *M. tuberculosis* strains.[4,5] The currently used live and attenuated *Mycobacterium bovis* Bacillus Calmette-Guérin (BCG) vaccine against tuberculosis faces several problems, which include variations in the protective efficacy, polymorphism in daughter strains, side effects in immuno-compromised patients, and the implication of misdiagnosis in vaccinated persons using tuberculin skin test, and so forth.[5-7] These limitations support the need of improving BCG, or developing alternative vaccines that are ultimately more effective against TB.[8] One approach to develop alternative vaccines is to clone and express immunodominant *M. tuberculosis*-specific proteins, deleted from BCG in non-pathogenic mycobacterial vehicles.[9,10] Genetic comparisons between *M. tuberculosis* and BCG have revealed several distinct genomic regions of differences (RD), including a 9.5 kb RD1 segment that is conserved in *M. tuberculosis,* but missing in all BCG strains, and most non-tuberculous mycobacteria.[11-13] The RD1 was shown to encode several secreted proteins with important immunological and protection implications, such as culture filtrate protein (CFP)10, and pulmonary embolism (PE) family-related protein PE35.[14-17] Antigen CFP10 elicits humoral and cellular immunity, and induces protection against *M. tuberculosis* infection in animals.[18-20] On the other hand, proteins of the PE family are known to induce protective immune responses during tuberculous infection in natural host species, and to have potentials in practical applications, as immunological markers of infection, and for the development of vaccines against tuberculosis.[21,22]

Compared with cytosolic and cell wall associated proteins, the secreted proteins are considered more efficient in the induction of protective immune responses.[23] Therefore, we selected the shuttle plasmid vector pDE22, which directs the expression of recombinant genes as secreted proteins.[24] The pDE22 vector, originating from pBLUE SCRIPT SKII polylinker and p16RI vector contains the hygromycin resistance gene marker, heat shock protein (Hsp)60 transcription signal, and secretion signal of the *M. tuberculosis* alpha antigen to direct the expression of recombinant genes as secreted proteins.[24] On the other hand, *M. vaccae* was selected as the vehicle for antigen delivery because of several useful properties of this species. First, *M. vaccae* is a non-pathogenic environmental, and relatively fast growing mycobacterium, which makes it suitable for laboratory manipulation and standardization. Second, *M. vaccae* has been used previously as a host for the expression of recombinant mycobacterial proteins, including superoxide dismutase, major extracellular proteins, and 19 kDa lipoprotein of *M. tuberculosis*.[25-27] In addition, *M. vaccae* has been proven to be an appropriate vehicle to deliver peptide epitopes, and to induce antigen-specific immune responses in animal models.[25-28] In this study, for the secretion of expressed proteins, we cloned PE35 and CFP10 genes in the expression vector pDE22, and the recombinant plasmids were used to transform *M. vaccae.* The expression of the cloned genes in recombinant *M. vaccae* (*rVaccae*) was determined by Western immunoblotting, and the induction of cellular and humoral immune responses to the expressed proteins were studied in rVaccae-immunized mice using splenocytes and sera in antigen-induced proliferation and antibody assays. Our objective is to clone and express *M. tuberculosis* - proteins PE35 and CFP10 in *M. vaccae*, and subsequently, evaluate the humoral and cellular immunity responses against these recombinant constructs in mice.

Methods. Experiments were carried out in the laboratory of the Faculty of Medicine, Kuwait University, Safat, Kuwait between 2009 and 2011.

Bacterial strains and plasmids. The *M. vaccae* was obtained from the American Type Culture Collection (ATCC 15483) (Rockville, MD, USA). Bacterial cells were grown in Middlebrook 7H9 medium supplemented with 2% glucose.[10,25] Plasmids were maintained in *rVaccae* by the addition of 50 μg/ml hygromycin (Invitrogen, Carlsbad, CA, USA). The construction of recombinant plasmids rpGES-TH1-PE35 and rpGES-TH1-CFP10, and the expression of recombinant proteins PE35 and CFP10 in *Escherichia coli* (*E. coli*) have been described previously.[19] The recombinant pDE22-PE35and pDE22-CFP10 vectors were constructed by double digestion of rpGES-TH1-PE35 and rpGES-TH1-CFP10 constructs using BamHI

and HindIII restriction enzymes, and subcloning of the released fragments into pDE22 shuttle vector predigested with the same restriction enzymes according to standard methods.[29,30] The recombinant pDE22 plasmids were propagated in *E. coli*, and identities of the DNA inserts were confirmed by polymerase chain reaction (PCR) amplification of PE35 and CFP10 DNA using gene-specific oligonucleotide primers (given below), and DNA sequencing according to the methods described previously.[24,29]

Oligonucleotides primers. The forward (F) and reverse (R) oligonucleotide primers for the amplification of PE35 and CFP10 genes by PCR were designed based on their nucleotide sequence in the *M. tuberculosis* H37Rv genome.[30] The DNA sequences of F and R primers for PE 35 and CFP 10 genes are shown below:

PE35 F 5'-AATCGG ATCCATGGAAAAAATGTCACATGATCCG-3'

PE35 R 5'-ACGAAGCTTTTCGGCGAAGA CGCCGGCGGCGCCGT-3'

CFP10 F 5'-AATCGGATCCATGG CAGAGATGAAGACCGATGCC-3'

CFP10 R 5'-ACGTAAGCTTGAAGCCC ATTTGCGAGGACAG-3'

Another set of F (pDE22F: 5'-AGCGTAAGTAGCGGGGTTG-3') and R (pDE22R: 5'- TGGCTTGTCCAAGGGTGTAT-3') primers were designed, which were based on the sequence of pDE22 shuttle vector, and covered its transcriptional and translational signals.[24] These primers were used in DNA sequencing to confirm the presence, and suitability of the inserts for expression in recombinant pDE22-PE35and pDE22-CFP10 vectors. All the primers were synthesized commercially (Interactiva Biotechnologies GmbH, Ulm, Germany).

Transformation of M. vaccae. The recombinant plasmids pDE22-PE35 and pDE22-CFP10 were isolated from *E. coli* cultures on a large scale using Maxiprep EndoFree Plasmid Purification Kits (QIAGEN, Valencia, CA, USA) according to manufacturer's instructions. Transformation of *M. vaccae* was carried out by electroporation using DNA (15-20 μg of each recombinant plasmid) and a Gene Pulser (BIO-RAD, Hercules, CA, USA) at the following settings: resistance 1000 ohms; capacitance 25 μFD; and voltage 2.5 V.[31] The colonies of transformed cells were isolated using standard procedures,[31] and cultured in 50 ml 7H9 medium for 10 days in the presence of 50 μg/ml hygromycin. The cells were harvested and washed with 0.9% saline, resuspended in 50 μl 1xTE (10 mM Tris-HCl containing 1 mM EDTA, pH 8.0), and boiled at 95°C for 25 minutes. To confirm the transformation of *M. vaccae* with the recombinant plasmids, 5 μl of the boiled samples were used in the PCR in the presence of gene-specific primers, and the products were analyzed by agarose gel electrophoresis, as described previously.[29]

Mitogen, mycobacterial sonicates, and synthetic peptides. The mitogen Concanavalin A (Con A) was purchased from Sigma Chemicals, St. Louis, MO, USA. To prepare *M. vaccae* sonicates, single colonies of wild-type and recombinant *M. vaccae* were grown in 50 ml 7H9 Middlebrook medium (Difco, Detroit, MI, USA), with or without hygromycin. Bacterial cells were harvested by centrifugation at 3500 x g for 15 minutes at 4°C, washed twice with 25 ml phosphate-buffered saline (PBS, pH 7.4), and resuspended in one ml sterile PBS. Bacterial cells were sonicated on ice using an XL Ultrasonic Processor (Heat Systems, NY, USA) with a standard, tapered microprobe tip, as described previously.[32] The protein concentrations in the sonicated supernatants were determined by measuring optical density (OD) at 280 nm. A total of 12 overlapping peptides (25-mers overlapping with neighboring peptides by 10 residues) covering the sequence of PE35 (P1 to P6), and CFP10 (P1 to P6) were synthesized commercially by Thermo Electron GmBH (Ulm, Germany) using fluorenylmethoxycarbonyl chemistry, as described previously.[22] The stock concentrations (5 mg/ml) of the peptides were prepared by dissolving peptide-powders in normal saline (0.9%) by vigorous pipetting, and the working concentrations were prepared in tissue culture medium RPMI-1640, as described previously.[14]

Analysis of recombinant protein expression in rVaccae. Mycobacterial sonicates were prepared as discussed earlier. In addition, proteins constitutively expressed in the supernatants of growing mycobacterial cultures were precipitated with 80% ammonium sulfate and re-dissolved in PBS (pH 7.4). Furthermore, sonicated *E. coli* preparations containing recombinant fusion proteins glutathione S-transferase (GST)-PE35 and GST-CFP10 were obtained upon expression of these proteins in *E. coli* transformed with the corresponding pGES-TH1 constructs, as described previously.[29] In all cases, proteins in bacterial cell sonicates and supernatants were analyzed by 15% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels, as described previously.[33] The resolved proteins were stained with Coomassie Brilliant Blue, or transferred to nitrocellulose membranes for Western immunoblotting using rabbit sera containing antigen-specific antibodies according to standard procedures.[19]

Ethical approval. Mice were immunized and handled according to established Institutional Animal Care and Use Committees approved protocols at Kuwait University, Kuwait.

Immunization of mice with native and recombinant M. vaccae. Six groups of 6 to 8-week-old female BALB/c mice (5 mice in each group) were included in the study. The first group of animals was non-immunized, and the second group was immunized with native *M. vaccae*. The animals of group 3 and group 4 were immunized with rVaccae-pDE22-PE35, and group 5 and group 6 with rVaccae-pDE22-CFP10. For all immunizations, the preparations of native *M. vaccae* and *rVaccae* were suspended in PBS + 0.05% Tween 80 (PBS-Tween), and 100 µl containing $5x10^8$ colony forming units (CFU) were injected intraperitoneally; boosters were given at 2 week intervals. All the mice of group 1 and group 2 were sacrificed 2 weeks after the primary immunization; whereas 2 animals each of the group 3 and group 5 were sacrificed after 2 weeks of the primary immunization, and the remaining animals were sacrificed 2 weeks after the first booster. The mice of group 4 and group 6 received 3 boosters, and all of them were sacrificed 2 weeks after the last immunization. Spleens and sera from all sacrificed mice were collected for further experiments, as given below.

Antigen and peptide-induced proliferation of mice splenocytes. Cells from each spleen were teased out and suspended at $5x10^6$/ml in complete tissue culture medium (RPMI-1640 medium supplemented with 20% fetal bovine serum, and $5x10^{-5}$ M ß-mercaptoethanol (ME) (Sigma, St. Louis, MO, USA), and were used in splenocytes proliferation assays according to standard procedures.[14] In brief, $2x10^5$ splenocytes were seeded in 96 well plates containing complete tissue culture medium and stimulated with mitogen ConA, *M. vaccae* sonicate, peptides pools, or overlapping individual peptides covering the sequence of each corresponding RD1 protein.[34-36] The cell cultures were incubated at 37^0C in a humidified atmosphere supplemented with 5% CO_2, pulsed on day 3 with one µCi ^{3}H-Thymidine (Amersham Life Science, Amersham, UK), and harvested 18 hours later on filter mats with Skatron harvester (Skatron Instruments AS, Oslo, Norway). The amount of incorporated radioactivity was determined using liquid scintillation ß plate counter (Wallac, Oy, Finland). Proliferation of splenocytes was considered positive if the stimulation index (SI) value was greater than 2.[37] The SI was defined as the average cpm in duplicate wells with antigen⁄average counts per minute (cpm) in duplicate wells without antigen.

Quantitation of T helper cell (Th)1 (interferon [INF]-γ), Th2 (interleukin [IL]-5), and anti-inflammatory (IL-10) cytokines secreted by mice splenocytes. Supernatants (100 µl) were collected from mice splenocytes cell cultures before being pulsed with ^{3}H-thymidine, and kept frozen until assayed for various cytokines. The 3 cytokines INF-γ, IL-5, and IL-10 were quantified by ELISA using commercial kits (Bender MedSystems, San Diego, CA, USA) according to the manufacturers' instructions. The 3 kits offered minimum detection limits of 5.3 for INF-γ, 5.0 for IL-10, and 3.3 pg/ml for IL-5. The cytokine response to a given antigen or peptide was considered positive when the concentration was greater than 100 pg/ml, and more than 2 times the concentration in control cultures without antigen stimulation.[35]

Enzyme linked immunosorbent assay (ELISA) for antibodies. The ELISA was performed to detect antibodies against the peptides pools and individual peptides of PE35 and CFP10 in the sera of pre- and post-immunized mice according to standard procedures.[38] In brief, wells of 96-well PolySorp plates (Nunc, Rochester, NY, USA) were coated with peptides (10 µg/ml), blocked with the blocking buffer, incubated with the primary antibody (mouse sera at 1:100) followed by secondary antibody (alkaline phosphatase-conjugated anti-rabbit immunoglobulin G), and addition of substrate for color development, as described previously.[39] The color intensity was determined by measuring the OD at 405 nm. The peptide-coated wells in the presence of secondary antibody alone, that is, without adding primary antibody were used as negative controls. The results were expressed as E/C, which is defined as:

$$E/C = \frac{\text{OD in peptide-coated wells with primary and secondary antibodies}}{\text{OD in peptide-coated wells with secondary antibody alone}}$$

The values of E/C >2.0 were considered positive.[19]

Results. ***Construction of recombinant M. vaccae.*** The DNA inserts of PE35 and CFP10 were restriction digested from rpGES-TH1-PE35 and rpGES-TH1-CFP10 and subcloned into pDE22. The identity of inserts in the resulting recombinant plasmids rpDE22-PE35 and rpDE22-CFP10 was confirmed by PCR and DNA sequencing (data not shown). The *M. vaccae* cells were transformed with rpDE22-PE35 and rpDE22-CFP10 using electroporation and the recombinant colonies of *M. vaccae* were selected on hygromycin-containing medium. The presence of appropriate plasmids in rVaccae was confirmed by PCR

using gene-specific primers. Results showed that DNA corresponding to the size of PE35 and CFP10 were amplified from the tested colonies of rVacae-pDE22-PE35 (**Figure 1A**) and rVaacae-pDE22-CFP10 (**Figure 1B**).

Expression of PE35 and CFP10 in recombinant M. vaccae. Rabbit sera containing anti-PE35 and anti-CFP10 were used as probes in Western immunoblots to detect the expression of PE35 and CFP10 proteins in cell sonicates and supernatants of cultured recombinant *M. vaccae*. In addition, recombinant proteins expressed from the rpGES-TH-1-PE35 and rpGES-TH-1-CFP10 vectors in *E. coli* were used as positive controls. The results showed that anti-PE35 and anti-CFP10 antibodies reacted with the corresponding recombinant proteins expressed in *E. coli* (**Figure 2**, lane G, data shown for PE35), and anti-PE35 antibodies reacted with a product in the supernatant of rVaccae-pDE22-PE35 at approximately 20 kDa (**Figure 2**, lane C), which corresponded to the expected size of recombinant PE35. This band was absent in the sonicates of rVaccae-pDE22-PE35, as well as in sonicates and supernatants of native *M. vaccae*, and *M. vaccae* transformed with the parent shuttle

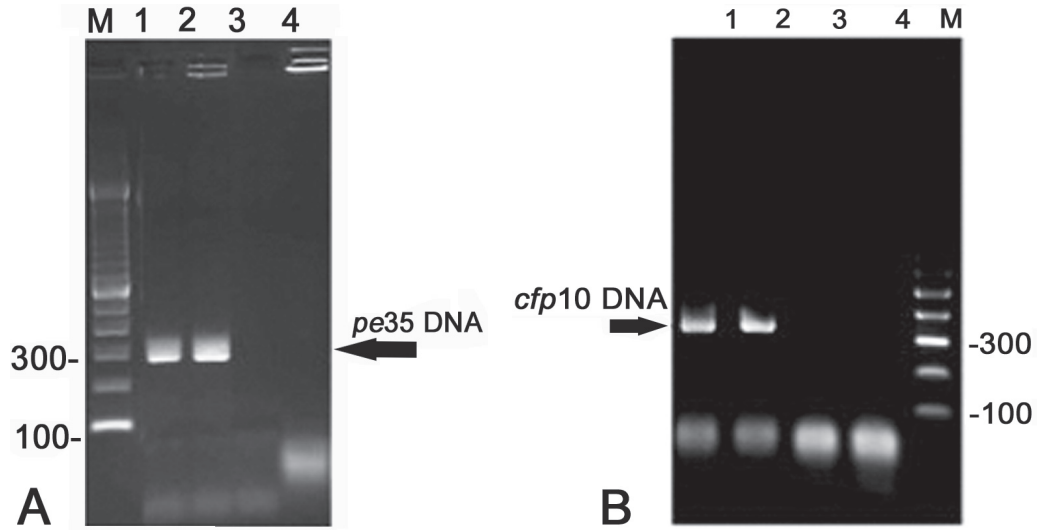

Figure 1 - Agarose gel electrophoresis of DNA amplified from recombinant *Mycobacterium vaccae* (rVaccae)-PDE22-PE35 and rVaccae-PDE22-CFP10 using: **A**) PE35-specific primers; and **B**) CFP10-specific primers. Panel A lane 1: PE35 DNA (300 bp) amplified from *Mycobacterium tuberculosis (M. tuberculosis)* H37Rv (positive control). Panel A, lane 2: PE35 DNA (300 bp) amplified from rVaccae-pDE22-PE35. Panel B, lane1: CFP10 DNA (303 bp) amplified from *M. tuberculosis* H37Rv (positive control). Panel B Lane 2: CFP10 DNA (303 bp) amplified from rVaccae-pDE22-CFP10. Panels A and B, lanes 3 and 4: No DNA amplified from native *Mycobacterium vaccae* and water (negative controls). Panels A and B, lane M: 100 base pair ladder. CFP-culture filtrate protein

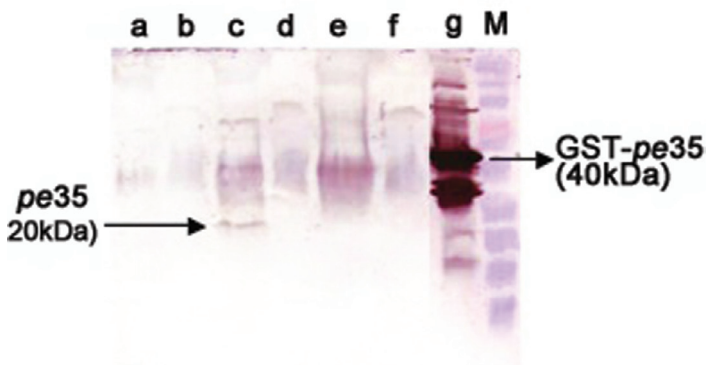

Figure 2 - Western immunoblotting to detect the expression of rPE35 protein in rVaccae-pDE22-PE35 using protein-specific antibodies: **a**) proteins from supernatants of native *Mycobacterium vaccae (M. vaccae*); **b**) proteins from sonicates of native *M. vaccae*; **c**) proteins from supernatants of rVaccae-pDE22-PE35 (long arrow shows a product at approximately 20 kDa reacting to anti-PE35 antibodies); **d**) proteins from sonicates of rVaccae-pDE22-PE35; **e**) proteins from supernatants of rVaccae-pDE22; **f**) proteins from sonicates of rVaccae-pDE22; **g**) proteins from sonicates of r*Escherichia coli* (*E. coli*)-pGES-TH1-PE35 (positive control), short arrow shows a product at approximately 40 kDa corresponding to fusion protein GST-PE35 in r*E. coli* sonicate reacting to the same antibodies; **M**) pre-stained low molecular weight protein marker.

Table 1 - Mitogen, antigen- and peptide-induced proliferation of splenocytes from non-immunized mice, and mice immunized with native *Mycobacterium vaccae (M. vaccae*), or rVaccae-pDE22-PE35.

Splenocytes stimulated with	Number of positive mice			
	Non-immunized mice (n=5)	Immunized mice		
		Native *M. vaccae* (n=5)	rVacce-pDE22-PE35	
			1 booster (n=3)	3 boosters (n=5)
ConA	5	5	3	5
M. vaccae sonicate	0	0	3	5
PE35 peptides pool	0	0	0	3
PE35 P1	0	0	0	1
PE35 P2	0	0	0	0
PE35 P3	0	0	0	3
PE35 P4	0	0	0	3
PE35 P5	0	0	0	1
PE35 P6	0	0	0	0

rVaccae - recombinant *M. vaccae*, ConA - Concanavalin A, P - peptide

Table 2 - Mitogen, antigen- and peptide-induced proliferation of splenocytes from non-immunized mice, and mice immunized with native *Mycobacterium vaccae (M. vaccae*), or rVaccae-pDE22-CFP10.

Splenocytes stimulated with	Number of positive mice			
	Non-immunized mice (n=5)	Immunized mice		
		Native *M. vaccae* (n=5)	rVacce-pDE22-CFP10	
			1 booster (n=3)	3 boosters (n=5)
ConA	5	5	3	5
M. vaccae sonicate	0	5	3	5
CFP10 peptides pool	0	0	0	4
P1	0	0	0	5
P2	0	0	0	0
P3	0	0	0	2
P4	0	0	0	2
P5	0	0	0	1
P6	0	0	0	1

rVaccae - recombinant *M. vaccae*, ConA - Concanavalin A, CFP - culture filtrate protein, P - peptide

vector pDE22 (**Figure 2**). However, the expression of recombinant CFP10 by Western immunoblotting was not detected in the culture supernatants, or the sonicates of rVaccae-pDE22-CFP10 (data not shown).

Antigen and peptide-induced proliferation of mice splenocytes. To study the expression and immunogenicity of PE35 and CFP10 in mice immunized with rVaccae constructs, splenocytes were used in antigen-induced proliferation assays. Splenocytes from non-immunized and native *M. vaccae*-immunized mice were used as controls. The splenocytes from all groups of mice were stimulated with the mitogen ConA, *M. vaccae* sonicate, pools of peptides of PE35 and CFP10, and the individual peptides. The results showed that splenocytes from all groups of mice (immunized and non-immunized) proliferated in response to stimulation with the mitogen ConA (SI ≥2) (**Table 1** & **Table 2**). However, proliferation in response to *M. vaccae* sonicate was not observed in non-immunized mice but splenocytes from mice immunized with native and recombinant *M. vaccae* proliferated to *M. vaccae* sonicate at all-time points after immunization (**Table 1** & **Table 2**). On the other hand, positive proliferation responses to peptides pools, and individual peptides of PE35 and CFP10 were observed only in mice receiving 3 booster injections of rVaccae (**Table 1** & **Table 2**). In the case of mice immunized with rVaccae-pDE22-PE35, peptides pool of PE35 induced positive proliferation in 3/5 mice, and the individual peptides P1, P3, P4, and P5 induced positive proliferation of splenocytes in 1/5, 3/5, 3/5, and 1/5 mice (**Table 1**); whereas, among mice immunized with rVaccae-pDE22-CFP10, 4/5 mice responded to the peptides pool of CFP10, and 5/5 (P1), 2/5 (P3), 2/5 (P4), 1/5 (P5), and 1/5 (P6) mice responded to the individual peptides (**Table 2**).

Antigen-induced secretion of cytokines by splenocytes of immunized mice. Splenocytes from all groups of animals secreted INF-γ, IL-5, and IL-10 in response to

Table 3 - Antibody reactivity to *Mycobacterium vaccae (M. vaccae*), sonicate, peptides pool, and individual peptides of PE35 in sera of mice immunized 4 times with rVaccae-pDE22/PE35.

Serum from mouse no.	Antibody reactivity (E/C)[a]							
	M. vaccae sonicate	PE35 peptides pool	P1	P2	P3	P4	P5	P6
1	16.8	2.5	0.9	2.4	1.0	1.0	0.8	1.3
2	8.0	1.6	0.9	1.5	0.9	0.9	0.7	0.9
3	7.6	2.0	1.9	2.7	0.7	0.9	0.7	0.8
4	14.9	1.6	1.0	1.9	0.9	0.9	0.8	1.1
5	16.2	1.3	0.9	1.9	0.7	0	0.7	1.0

rVaccae - recombinant *M. vaccae*, P - peptide

ConA, whereas only INF-γ and IL-10 were secreted in response to *M. vaccae* sonicate. However, none of the peptides of PE35 and CFP10 induced secretion of any tested cytokine from splenocytes of animals immunized with corresponding recombinant *M. vaccae* constructs (data not shown).

Humoral immune response in immunized mice against recombinant proteins. To study humoral responses in mice, sera were collected from the non-immunized and immunized mice, and tested for antibodies to *M. vaccae* sonicate, peptides pools and individual peptides of PE35 and CFP10 using ELISA. The results showed that positive antibody responses to *M. vaccae* sonicate were observed only in immunized mice injected with native or rVaccae after primary, as well as booster injections (**Table 3**, data shown for mice given 3 boosters). However, antibodies to peptides pools and individual peptides of PE35 and CFP10 proteins were not detected in sera from non-immunized mice, or mice immunized with native *M. vaccae* (data not shown). When tested with peptide pools and individual peptides of PE35 and CFP10, sera from 2/5 mice receiving 3 boosters with rVaccae-pDE22-PE35 showed positive antibody responses (E/C >2) to the peptides pool, and the individual peptide P2 of PE35 protein (**Table 3**), but mice immunized with rVaccae-pDE22-CFP10 did not show positive antibody responses to the peptides pool, or individual peptides of CFP10, even after 3 boosters (data not shown).

Discussion. In this study, we attempted to express 2 *M. tuberculosis*-specific and immunodominant RD1 proteins, that is, PE35 and CFP10 in non-pathogenic *M. vaccae* for immunological evaluation in mice. Both of these proteins are secreted naturally by *M. tuberculosis* during in vitro growth,[40,41] and are considered major T-cell antigens for the induction of protective immune responses against *M. tuberculosis*.[14,42] To mimic the natural expression, the coding DNA sequences of PE35 and CFP10 proteins of *M. tuberculosis* were cloned in the shuttle vector pDE22. This vector contains the secretion sequence motif of *M. tuberculosis* alpha antigen, which is expected to drive the secretion of the cloned recombinant proteins to the outside milieu.[24] Non-pathogenic *M. vaccae* was transformed with recombinant pDE22, and selected on hygromycin-containing medium because pDE22 has hygromycin resistance gene.[24] In addition to the known adjuvant properties of mycobacteria, *M. vaccae* was chosen as a vehicle for antigen delivery because of many other characteristics that this mycobacterium, particularly, possesses. First, *M. vaccae* is an environmental, non-pathogenic, and relatively fast grower (it grows in 10 days compared with 4 weeks for BCG), which makes it suitable for experimental standardizations and manipulations. Second, *M. vaccae* has the ability to allow stable maintenance and expression of heterologous DNA, which is an essential characteristic of a recombinant host.[43-45] In addition, *M. vaccae* modulates the immune response against tuberculosis in favor of protective Th1-type,[46,47] and whole heat-killed *M. vaccae* has shown promise as a clinical immunotherapeutic agent for TB treatment.[45,48]

The expression of the recombinant proteins in rVaccae constructs was determined by Western immunoblotting using rabbit sera containing antigen-specific antibodies.[19] Since recombinant proteins in rVaccae were expected to be actively secreted out by the bacterial cells, proteins present in culture supernatants, in addition to cell sonicates were used in Western immunoblots. Furthermore, sonicates of recombinant *E. coli* cells containing recombinant PE35 and CFP10 were used as positive controls.[19] The results showed that anti-PE35 and anti-CFP10 antibodies reacted with the corresponding recombinant proteins expressed in *E. coli* with a strong signal, suggesting that *E. coli* expressed a large quantity of these proteins. However, the results with rVaccae/pDE22-PE35 suggested a low level of expression of PE35 because anti-PE35 antibodies showed a faint product at approximately 20 kDa in the culture supernatant, but not in the cell sonicate of rVaccae/pDE22-PE35 (**Figure 2**). However, this reactivity was specific because no antibody reactivity with a protein of the expected size was observed in either supernatants, or sonicates of native *M. vaccae*, or recombinant *M. vaccae* transformed with parent pDE22 (**Figure 2**). The size of the detected protein in the supernatants of rVaccae/pDE22-PE35 was 20 kDa, which is close to the expected size of the recombinant PE35 expressed from pDE22-PE35, that is, 18 kDa, 10 kDa for PE35,[30] and 8 kDa for the secretion signal motif from the shuttle vector.[24] On the other hand, the expression of CFP10 in rVaccae-pDE22-CFP10 could not be confirmed by Western immunoblotting, which could be due to very low level of expression. Apparently, it was not due to any problem with the cloned DNA because DNA sequencing showed that both PE35 and CFP10 DNA inserts had 100% matching with the expected sequence in *M. tuberculosis* genome,[30] and both the inserts were in the frame with the promoter and intervening sequences.

To evaluate for the induction of antigen-specific immunological responses, BALB/c mice were immunized with rVaccae/pDE22-PE35 and rVaccae/

pDE22-CFP10 constructs. Cellular immune responses were evaluated using splenocytes in proliferation assays, which indicate T helper-1 type response, and correlate with protective immunity.[42] The results showed that positive proliferation responses to peptide pools covering the sequence of PE35 and CFP10 were observed in mice only after 3 booster immunizations. However, positive responses to *M. vaccae* sonicate and the T-cell mitogen ConA were observed with splenocytes of all mice after the first immunization, and as expected, the splenocytes from non-immunized mice proliferated to ConA only (**Table 1** & **Table 2**). These results suggest that the lack of splenocytes proliferation to the peptides of PE35 and CFP10 in mice receiving less than 3 boosters was not due to technical reasons. Furthermore, positive cellular responses to peptides pools of PE35 and CFP10 in mice receiving 3 boosters with rVaccae, suggests that both PE35 and CFP10 were expressed in rVaccae, but most probably at low levels, which necessitated multiple injections to sensitize sufficient number of cells to lead to a positive response. The low level expression may also be the reason for the fact that Western immunoblotting could not detect the expression of CFP10 in the culture supernatants of in vitro grown rVaccae-pDE22-CFP10.

To confirm the results obtained with the peptides pools and determine the number of epitopes inducing a positive cellular response, splenocytes from non-immunized and immunized mice were tested with individual peptides of PE35 and CFP10. The results showed that non-immunized and mice receiving less than 3 boosters did not respond to any of these peptides. However, splenocytes from mice receiving 3 boosters responded to 4 and 5 of the 6 peptides of PE35 and CFP10. These results confirm that the responses to peptide pools were not experimental artifacts, and several epitopes of these proteins were involved, as has been previously shown in *M. tuberculosis*-infected humans and guinea-pigs and mice immunized with DNA vaccine constructs of these proteins.[14,49-51] Furthermore, bioinformatics analysis, based on binding to HLA-DR molecules, has also predicted the presence of several T-cell epitopes in these proteins.[30,52] Interestingly, splenocytes from BALB/c mice immunized with a DNA vaccine construct of PE35 (pUMVC6/PE35) also responded to the same peptides of PE35, that is, P1, P3, P4, and P5 [14], as found in this study (**Table 1**).

In addition to cellular immune responses, humoral immune responses have been recently shown to contribute to protective immunity against TB.[53-55] The induction of humoral immune responses to recombinant antigens was evaluated by measuring antibodies in ELISA using sera from mice. Like cellular responses, antigen-specific antibodies were detected only in sera of mice that received 3 boosters with rVaccae/pDE22-PE35. However, only 2 of 5 mice in this group contained a positive antibody titer to the peptide pool of PE35, and to its individual peptide P2 (**Table 3**). On the other hand, the absence of detectable levels of anti-CFP10 antibodies in the sera of mice immunized with rVaccae/pDE22-CFP10 could again be due to very low expression level of protein. Alternatively, the *M. vaccae*-derived protein is subject to post translational modifications, and it is possible that lipid or carbohydrate moieties influence its immune recognition, and thus, the nature of antibodies induced,[43,44] which may not be recognized by overlapping peptides used in this work.

The expression of TB proteins in recombinant *M. vaccae* is an attractive strategy for vaccine production. However, according to the results of this and other studies,[44] low level expression of the recombinant proteins imposes an important limitation on the development of this approach. To obtain high-level expression, other expression systems should be tested, such as the system based on the iron-containing superoxide dismutase (SOD) of *M. tuberculosis*, as a carrier protein. The gene encoding SOD can be expressed at high levels in recombinant mycobacterial systems, accounting for as much as 10% of the total cell protein.[31,44]

In conclusion, this study confirms the in vitro and in vivo expression of PE35, as well as immunogenicity of both PE35 and CFP10 recombinant proteins in *M. vaccae*; however, the expression is low.

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References

1. Cramm JM, Finkenflügel HJ, Møller V, Nieboer AP. TB treatment initiation and adherence in a South African community influenced more by perceptions than by knowledge of tuberculosis. ***BMC Public Health*** 2010; 10: 72.
2. World Health Organization. WHO Report 2010. Global Tuberculosis Control. Available from: http://www.who.int/tb/publications/global_report/2010/en/
3. Ducati RG, Ruffino-Netto A, Basso LA, Santos DS. The resumption of consumption - a review on tuberculosis. ***Mem Inst Oswaldo Cruz*** 2006; 101: 697-714.
4. Liu J, Tran V, Leung AS, Alexander DC, Zhu B. BCG vaccines: Their mechanisms of attenuation and impact on safety and protective efficacy. ***Hum Vaccin*** 2009; 5: 70-78.
5. Svenson S, Källenius G, Pawlowski A, Hamasur B. Towards new tuberculosis vaccines. ***Hum Vaccin*** 2010; 6: 309-317.
6. Flynn JL. Immunology of tuberculosis and implications in vaccine development. ***Tuberculosis (Edinb)*** 2004; 84: 93-101.

7. Mustafa AS. What's new in the development of tuberculosis vaccines. ***Med Princ Pract*** 2012; 21: 195-196.
8. Mustafa AS, Al-Attiyah R. Tuberculosis: looking beyond BCG vaccines. ***J Postgrad Med*** 2003; 49: 134-140.
9. Kaufmann SH, Gengenbacher M. Recombinant live vaccine candidates against tuberculosis. ***Curr Opin Biotechnol*** 2012; 23: 900-907.
10. Medeiros MA, Dellagostin OA, Armôa GR, Degrave WM, De Mendonça-Lima L, Lopes MQ, et al. Comparative evaluation of *Mycobacterium vaccae* as a surrogate cloning host for use in the study of mycobacterial genetics. ***Microbiology*** 2002; 148: 1999-2009.
11. Behr MA, Wilson MA, Gill WP, Salamon H, Schoolnik GK, Rane S, et al. Comparative genomics of BCG vaccines by whole-genome DNA microarray. ***Science*** 1999; 284: 1520-1523.
12. Mahairas GG, Sabo PJ, Hickey MJ, Singh DC, Stover CK. Molecular analysis of genetic differences between *Mycobacterium bovis* BCG and virulent *M. bovis*. ***J Bacteriol*** 1996; 178: 1274-1282.
13. Amoudy HA, Mustafa AS. Amplification of six putative RD1 genes of *Mycobacterium tuberculosis* for cloning and expression in *Escherichia coli* and purification of expressed proteins. ***Med Princ Pract*** 2008; 17: 378-384.
14. Hanif SN, Al-Attiyah R, Mustafa AS. Cellular immune responses in mice induced by *M. tuberculosis* PE35-DNA vaccine construct. ***Scand J Immunol*** 2011; 74: 554-560.
15. Mustafa AS, Al-Attiyah R, Hanif SN, Shaban FA. Efficient testing of large pools of *Mycobacterium tuberculosis* RD1 peptides and identification of major antigens and immunodominant peptides recognized by human Th1 cells. ***Clin Vaccine Immunol*** 2008; 15: 916-924.
16. Mustafa AS. Th1 cell reactivity and HLA-DR binding prediction for promiscuous recognition of MPT63 (Rv1926c), a major secreted protein of *Mycobacterium tuberculosis*. ***Scand J Immunol*** 2009; 69: 213-222.
17. Mustafa AS. Comparative evaluation of MPT83 (Rv2873) for T helper-1 cell reactivity and identification of HLA-promiscuous peptides in *Mycobacterium bovis* BCG-vaccinated healthy subjects. ***Clin Vaccine Immunol*** 2011; 18: 1752-1759.
18. Guo S, Xue R, Li Y, Wang SM, Ren L, Xu JJ. The CFP10/ESAT6 complex of *Mycobacterium tuberculosis* may function as a regulator of macrophage cell death at different stages of tuberculosis infection. ***Med Hypotheses*** 2012; 78: 389-392.
19. Hanif SN, Al-Attiyah R, Mustafa AS. Molecular cloning, expression, purification and immunological characterization of three low-molecular weight proteins encoded by genes in genomic regions of difference of *Mycobacterium tuberculosis*. ***Scand J Immunol*** 2010; 71: 353-361.
20. Cockle PJ, Gordon SV, Lalvani A, Buddle BM, Hewinson RG, Vordermeier HM. Identification of novel *Mycobacterium tuberculosis* antigens with potential as diagnostic reagents or subunit vaccine candidates by comparative genomics. ***Infect Immun*** 2002; 70: 6996-7003.
21. Delogu G, Brennan MJ. Comparative immune response to PE and PE_PGRS antigens of *Mycobacterium tuberculosis*. ***Infect Immun*** 2001; 69: 5606-5611.
22. Mustafa AS, Cockle PJ, Shaban F, Hewinson RG, Vordermeier HM. Immunogenicity of *Mycobacterium tuberculosis* RD1 region gene products in infected cattle. ***Clin Exp Immunol*** 2002; 130: 37-42.
23. Mustafa AS. Mycobacterial gene cloning and expression, comparative genomics, bioinformatics and proteomics in relation to the development of new vaccines and diagnostic reagents. ***Med Princ Pract*** 2005; 14 (Suppl 1): 27-34.
24. Gaora P, Barnini S, Hayward C, Filley E, Rook G, Young D, et al. Mycobacteria as immunogens: Development of expression vectors for use in multiple *Mycobacterial* species. ***Med Princ Pract*** 1997; 6: 91-96.
25. Abou-Zeid C, Gares MP, Inwald J, Janssen R, Zhang Y, Young DB, et al. Induction of a type-1 immune response to a recombinant antigen from *Mycobacterium tuberculosis* expressed in *Mycobacterium vaccae*. ***Infect Immun*** 1997; 65: 1856-1862.
26. Janssen R, Kruisselbrink A, Hoogteijling L, Lamb JR, Young DB, Thole JE. Analysis of recombinant mycobacteria as T helper type 1 vaccines in an allergy challenge model. ***Immunology*** 2001; 102: 441-449.
27. Gao H, Bai Y, Xue Y, Wang L, Fan A, Ding X, et al. Expression, purification, and characterization of soluble RpfD with high bioactivity as a recombinant protein in *Mycobacterium vaccae*. ***Protein Expr Purif*** 2007; 55: 112-118.
28. Yeremeev VV, Lyadova IV, Nikonenko BV, Apt AS, Abou-Zeid C, Inwald J, et al. The 19-kD antigen and protective immunity in a murine model of tuberculosis. ***Clin Exp Immunol*** 2000; 120: 274-279.
29. Ahmad S, El-Shazly S, Mustafa AS, Al-Attiyah R. Mammalian cell-entry proteins encoded by the mce3 operon of *Mycobacterium tuberculosis* are expressed during natural infection in humans. ***Scand J Immunol*** 2004; 60: 382-391.
30. Shaban K, Amoudy HA, Mustafa AS. Cellular immune responses to recombinant *Mycobacterium bovis* BCG constructs expressing major antigens of region of difference 1 of *Mycobacterium tuberculosis*. ***Clin Vaccine Immunol*** 2013; 8: 1230-1237.
31. Garbe TR, Barathi J, Barnini S, Zhang Y, Abou-Zeid C, Tang D, et al. Transformation of mycobacterial species using hygromycin resistance as selectable marker. ***Microbiology*** 1994; 140: 133-138.
32. Lanigan MD, Vaughan JA, Shiell BJ, Beddome GJ, Michalski WP. Mycobacterial proteome extraction: comparison of disruption methods. ***Proteomics*** 2004; 4: 1094-1100.
33. Ahmad S, Akbar PK, Wiker HG, Harboe M, Mustafa AS. Cloning, expression and immunological reactivity of two mammalian cell entry proteins encoded by the mce1 operon of *Mycobacterium tuberculosis*. ***Scand J Immunol*** 1999; 50: 510-518.
34. Mustafa AS. Recombinant and synthetic peptides to identify *Mycobacterium tuberculosis* antigens and epitopes of diagnostic and vaccine relevance. ***Tuberculosis (Edinb)*** 2005; 85: 367-376.
35. Al-Attiyah R, Mustafa AS. Characterization of human cellular immune responses to novel *Mycobacterium tuberculosis* antigens encoded by genomic regions absent in *Mycobacterium bovis* BCG. ***Infect Immun*** 2008; 76: 4190-4198.
36. Mustafa AS, Al-Saidi F, El-Shamy AS, Al-Attiyah R. Cytokines in response to proteins predicted in genomic regions of difference of *Mycobacterium tuberculosis*. ***Microbiol Immunol*** 2011; 55: 267-278.
37. Al-Attiyah R, Mustafa AS. Characterization of human cellular immune responses to novel *Mycobacterium tuberculosis* antigens encoded by genomic regions absent in *Mycobacterium bovis* BCG. ***Infect Immun*** 2008; 76: 4190-4198.
38. Amoudy HA, AI-Asmer ABH, Abul AT, Mustafa AS. Evaluation of complex and defined antigens of *Mycobacterium tuberculosis* in an IgG specific ELISA for the diagnosis of tuberculosis. ***Med Principles Pract*** 1997; 6: 103-109.

39. El-Shazly S, Mustafa AS, Ahmad S, Al-Attiyah R. Utility of three mammalian cell entry proteins of Mycobacterium tuberculosis in serodiagnosis of tuberculosis. ***Int J Tuberc Lung Dis*** 2007; 11: 676-682.
40. Målen H, Berven FS, Fladmark KE, Wiker HG. Comprehensive analysis of exported proteins from *Mycobacterium tuberculosis* H37Rv. ***Proteomics*** 2007; 7: 1702-1718.
41. Målen H, Søfteland T, Wiker HG. Antigen analysis of *Mycobacterium tuberculosis* H37Rv culture filtrate proteins. ***Scand J Immunol*** 2008; 67: 245-252.
42. Mustafa AS. Vaccine potential of *Mycobacterium tuberculosis*-specific genomic regions: in vitro studies in humans. ***Expert Rev Vaccines*** 2009; 8: 1309-1312.
43. Harth G, Lee BY, Horwitz MA. High-level heterologous expression and secretion in rapidly growing non-pathogenic mycobacteria of four major *Mycobacterium tuberculosis* extracellular proteins considered to be leading vaccine candidates and drug targets. ***Infect Immun*** 1997; 65: 2321-2328.
44. Hetzel C, Janssen R, Ely SJ, Kristensen NM, Bunting K, Cooper JB, et al. An epitope delivery system for use with recombinant mycobacteria. ***Infect Immun*** 1998; 66: 3643-2648.
45. Xu LJ, Wang YY, Zheng XD, Gui XD, Tao LF, Wei HM. Immunotherapeutical potential of *Mycobacterium vaccae* on *M. tuberculosis* infection in mice. ***Cell Mol Immunol*** 2009; 6: 67-72.
46. Rodríguez-Güell E, Agustí G, Corominas M, Cardona PJ, Luquin M, Julián E. Mice with pulmonary tuberculosis treated with *Mycobacterium vaccae* develop strikingly enhanced recall gamma interferon responses to *M. vaccae* cell wall skeleton. ***Clin Vaccine Immunol*** 2008; 15: 893-896.
47. Skinner MA, Yuan S, Prestidge R, Chuk D, Watson JD, Tan PL. Immunization with heat-killed *Mycobacterium vaccae* stimulates CD8+ cytotoxic T cells specific for macrophages infected with *Mycobacterium tuberculosis*. ***Infect Immun*** 1997; 65: 4525-4530.
48. Dlugovitzky D, Fiorenza G, Farroni M, Bogue C, Stanford C, Stanford J. Immunological consequences of three doses of heat-killed *Mycobacterium vaccae* in the immunotherapy of tuberculosis. ***Respir Med*** 2006; 100: 1079-1087.
49. Hanif SN, Al-Attiyah R, Mustafa AS. DNA vaccine constructs expressing *Mycobacterium tuberculosis*-specific genes induce immune responses. ***Scand J Immunol*** 2010; 72: 408-415.
50. Hanif SN, Al-Attiyah R, Mustafa AS. Species-specific antigenic *Mycobacterium tuberculosis* proteins tested by delayed-type hypersensitivity response. ***Int J Tuberc Lung Dis*** 2010; 14: 489-494.
51. Hanif SN, El-Shammy AM, Al-Attiyah R, Mustafa AS. Whole blood assays to identify Th1 cell antigens and peptides encoded by *Mycobacterium tuberculosis*-specific RD1 genes. ***Med Principles Pract*** 2008; 17: 244-249.
52. Mustafa AS, Shaban FA. ProPred analysis and experimental evaluation of promiscuous T-cell epitopes of three major secreted antigens of *Mycobacterium tuberculosis*. ***Tuberculosis (Edinb)*** 2006; 86: 115-124.
53. Zuñiga J, Torres-García D, Santos-Mendoza T, Rodriguez-Reyna TS, Granados J, Yunis EJ. Cellular and humoral mechanisms involved in the control of tuberculosis. ***Clin Dev Immunol*** 2012; 2012: 193923.
54. Acosta A, Norazmi MN, Sarmiento ME. Antibody mediated immunity - a missed opportunity in the fight against tuberculosis? ***Malays J Med Sci*** 2010; 17: 66-67.
55. Abebe F, Bjune G. The protective role of antibody responses during Mycobacterium tuberculosis infection. ***Clin Exp Immunol*** 2009; 157: 235-243.

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Dong M, Wang D, Jiang Y, Zhao L, Yang C, Wu C. In vitro efficacy of acetohydroxyacid synthase inhibitors against clinical strains of *Mycobacterium tuberculosis* isolated from a hospital in Beijing, China. ***Saudi Med J*** 2011; 32: 1122-1126.

Mottalib A, Hossain M, Khalil I, Islam S, Hossain A. Drug susceptibility pattern of *Mycobacterium tuberculosis* isolates against conventional anti-tuberculosis drugs in Dhaka, Bangladesh. ***Saudi Med J*** 2011; 32: 484-488.

Abdel-Aziz NA, Al-Harbi KM, Morsy MF, Turkistani KA, Kurdi FN. Evaluation of direct detection of *Mycobacterium tuberculosis* in clinical samples using the BD ProbeTec ET system. ***Saudi Med J*** 2011; 32: 123-127.