

## The Natural Expression of Genes Encoding Major Antigens of Rd1 and Rd9 in *M. Tuberculosis* and other Mycobacteria

Shumaila N. M. Hanif\*, Rajaa Al-Attayah and Abu Salim Mustafa

Department of Microbiology, Faculty of Medicine, Kuwait University, Kuwait

### Abstract

The natural expression of genes of five major antigenic proteins present in the genomic region of difference (RD)1, i.e. *pe35*, *ppeE68*, *esxA* and *esxB*, and RD9, i.e. *esxV*, was determined, at mRNA and protein levels, in *M. tuberculosis*, *M. bovis* BCG, and environmental mycobacterial species of *M. avium* and *M. vaccae*. The expression of mRNA was determined using total mycobacterial RNA and gene-specific forward and reverse primers in reverse transcription-polymerase chain reaction (RT-PCR), and the expression of proteins was determined by Western immunoblot analysis of mycobacterial sonicates with anti-sera containing protein-specific antibodies raised in rabbits. Furthermore, the expression of each protein in various sub-cellular fractions of *M. tuberculosis* was studied by Western immunoblot analysis of cell walls, culture filtrates and sonicates of *M. tuberculosis* using the rabbit anti-sera. The results showed mRNA and protein expressions for PE35, PPE68, ESXA, and ESXB only in *M. tuberculosis*, whereas ESXV was expressed in *M. tuberculosis* and *M. bovis* BCG, but none of them were expressed in *M. avium* and *M. vaccae*. Furthermore, all of the proteins were detected in various sub-cellular fractions of *M. tuberculosis*. In conclusion, our study demonstrates that the five major antigenic proteins encoded by genes present in RD1 and RD9 regions are naturally expressed in *M. tuberculosis*, whereas only ESXV is expressed in *M. bovis* BCG and none of these proteins are expressed in the environmental mycobacterial species of *M. avium* and *M. vaccae*. As these proteins are major antigens recognized by Th1 cells of protective phenotype, they could be useful in specific diagnosis and/or development of new vaccines against tuberculosis.

**Keywords:** Tuberculosis; RD1; RD9; Major Antigens; Expression; Mycobacteria

### Introduction

Tuberculosis (TB) is a major global health problem with 1/3<sup>rd</sup> of the world population infected with *Mycobacterium tuberculosis*, about 9.3 million people developing active disease leading to 1.8 million deaths annually [1]. The threat posed by TB to human health is increasing due to emergence and world-wide spread of multi and excessive drug-resistant strains of *M. tuberculosis* and co-infections with human immunodeficiency virus (HIV) [1]. It is suggested that the effective control of TB may only be feasible if appropriate new tools become available for specific diagnosis and vaccination [2].

The current immunodiagnosis of TB is based on the use of purified protein derivative (PPD) of *M. tuberculosis*, as a tuberculin skin test. However, due to antigenic cross-reactivity with other mycobacteria, a positive tuberculin test may not differentiate between people with active/latent TB, BCG vaccination, or cross-sensitization with environmental mycobacterial species [3]. The only available vaccine against TB is BCG, a live attenuated strain of pathogenic *Mycobacterium bovis*, developed by Calmette and Guérin in 1921 [4]. BCG has been widely used to vaccinate against TB, and has shown about 50% efficacy in children and against extra-pulmonary disease, but it has completely failed in adults against pulmonary disease, which is the major clinical manifestation of TB [5]. In addition, BCG vaccination can cause disseminated disease in immunocompromised individuals [6] and compromises the diagnostic efficacy of PPD due to the presence of crossreactive antigens [5]. These limitations of PPD and BCG have generated interest in the identification of species-specific and immunodominant antigens of *M. tuberculosis* for applications in diagnosis and development of new vaccines against TB [7,8].

The sequencing of complete genome of *M. tuberculosis* and its comparison with other mycobacteria has identified 11 genomic regions of difference (RD) that are deleted in all vaccine strains of *M. bovis*

BCG i.e. RD1, RD4 to RD7, RD9 to RD13 and RD15 [9]. Testing of overlapping synthetic peptides corresponding to proteins encoded by genes predicted in these regions has identified RD1 and RD9 as regions having immunodominant antigens recognized by protective Th1 cells [10,11]. Further testing of individual proteins encoded by these regions identified PE35, PPE68, ESXA and ESXB of RD1 and ESXV of RD9 as major antigens in TB patients [12-15]. However, PPE68 and ESXV were also recognized as major antigens in BCG-vaccinated healthy subjects [12,15]. This could have been due to the presence of these genes or their homologs in BCG and/or environmental mycobacteria. The present study was conducted to determine if, in addition to *M. tuberculosis*, these genes are present and expressed in BCG and two species of environmental mycobacteria i.e. *M. avium* and *M. vaccae* at the level of mRNA and proteins by using gene-specific primers in reverse-transcription polymerase chain reaction (RT-PCR) and anti-protein antibodies in Western immunoblotting, respectively.

### Materials and Methods

#### Mycobacterial sonicates, culture filtrate and cell walls

*M. tuberculosis* H<sub>37</sub>Rv, *M. bovis* BCG, *M. avium*, and *M. vaccae*

**\*Corresponding author:** Shumaila Nida Muhammad Hanif, Department of Microbiology, Faculty of Medicine, Kuwait University, P.O. BOX 24923 13110, Safat, Kuwait. Telephone: 00965-97542085; Fax: 00965-25332719; E-mail: [dr.shumaila\\_nida@yahoo.com](mailto:dr.shumaila_nida@yahoo.com)

**Received** November 28, 2011; **Accepted** December 13, 2011; **Published** December 15, 2011

**Citation:** Hanif SNM, Al-Attayah R, Mustafa AS (2011) The Natural Expression of Genes Encoding Major Antigens of Rd1 and Rd9 in *M. Tuberculosis* and other Mycobacteria. Mycobact Diseases 1:105. doi:[10.4172/2161-1068.1000105](https://doi.org/10.4172/2161-1068.1000105)

**Copyright:** © 2011 Hanif SNM, et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

(obtained from the American Type Culture Collection, Rockville, MD, USA) were grown in Middlebrook 7H9 medium (Difco, Detroit, MI, USA) and sonicated as previously described [16]. *M. tuberculosis* culture filtrate (MT-CF) and cell walls (MT-CW) (provided by Dr. K. Dobos, Colorado State University, USA) were produced as part of NIH, NIAID Contract No. HHSN266200400091C entitled "Tuberculosis Vaccine Testing and Research Materials", which was awarded to Colorado State University.

### Synthetic peptides

Synthetic peptides (25-mers overlapping neighbouring peptides by 10 amino acids) covering the sequence of RD1 and RD9 proteins were designed based on their amino acid sequence deduced from the nucleotide sequence of the respective genes [10,17]. These peptides were commercially synthesized by Thermo Hybaid GmbH (Ulm, Germany) using fluorenylmethoxycarbonyl chemistry, as described previously [18-20]. The stock concentrations (5 mg/ml) of the peptides were prepared in normal saline (0.9%) by vigorous pipetting, and the working concentrations were prepared by further dilution in phosphate buffered saline (PBS, pH 7.0) as described previously [21,22].

### Oligonucleotide primers

The DNA sequences of forward (F) and reverse (R) primers for the amplification of *pe35*, *ppe68*, *esxA*, *esxB* and *esxV* genes, based on the N- and C-terminals of these genes in *M. tuberculosis* H37Rv [17] are shown below:

PE32 F: 5'-AATCGGATCCATGGAAAAATGTCACATGATCCG-3'

PE32 R: 5'-ACGAAGCTTTTCGGCGAAGACGCCGGCGGC-GCCGT-3'

PPE68 F: 5'-AATCGGATCCATGCTGTGCGACGCAATGC-CACCG-3'

PPE68 R: 5'-ACGAAGCTTCCAGTCGTCCTCTTC-GTCCAGT-3'

ESXB F: 5' AATCGGATCCATGGCAGAGATGAAGACCGATGCC3'

ESXB R: 5'-ACGAAGCTTGAAGCCATTTGCGAGGACAG -3'

ESXA F: 5'-AATCGGATCCATGACAGAGCAGCAGTG-GAATTTC -3'

ESXA R: 5'-ACGAAGCTTTGCGAACATCCCAGTGACGTT-3'

ESXV F: 5'AATCGGATCCATGACCATCAACTATCAATTG-GGGGAC3'

ESXV R: 5'ACGAAGCTTGGCCCAGCTGGAGCCGACGGC-GCT 3'

To facilitate gene cloning in plasmid vectors, additional sequences were added at the 5' ends of F and R primers, which contained *Bam*H I and *Hind* III restriction sites (bold face nucleotides), respectively.

### Isolation of RNA from mycobacteria and RT-PCR

Total cellular RNA were isolated from 14 days old cultures of mycobacterial cells and RT-PCR was performed according to procedures described previously [23]. In brief, the bacterial pellets were lysed and total nucleic acids were isolated using the MasterPure RNA purification kit (Epicenter Technologies, USA) according to the manufacturer's instructions. The contaminating DNA was digested

with 5µl of RNase-free DNase I solution (Epicenter Technologies, USA) for 30 min at 37°C. The reaction was stopped by adding 1µl of 25 mM ethylenediaminetetraacetic acid (EDTA) and the enzyme was inactivated at 75°C for 10 min. The isolated RNA was used to synthesize first-strand cDNA by reverse transcription using Not I-d(T)18 primer and other components included in the first-strand cDNA synthesis kit (Amersham Biosciences, UK) according to the manufacturer's instructions. The synthesized cDNA were amplified by PCR in a thermal cycler (Perkin Elmer System 9600) by using forward and reverse primers described above for each gene, according to standard procedures [23]. The same primers were used in PCR with genomic DNA and DNA-free RNA isolated from each mycobacterial species as positive and negative controls, respectively. A common mycobacterial target of 162 bp DNA was amplified by using primers MD1/MD2 to confirm the synthesis of cDNA from RNA of the tested mycobacterial species, as described previously [23]. To determine the amplification of the targets, aliquots (20µl) of the amplified products were analyzed by agarose gel electrophoresis, as described previously [24].

### Gene cloning, expression and protein purification

Full-length DNA corresponding to *pe35*, *pe68*, *esxA*, *esxB* and *esxV* were amplified by PCR using forward and reverse primers specific for each gene, as given above, and genomic DNA of *M. tuberculosis* H<sub>37</sub>Rv according to standard procedures [25]. The amplified DNA were cloned into pGEM-T Easy vector (Promega Corporation, Madison, WI, USA) and subcloned subsequently into pGES-TH-1 plasmid for the expression in *E. coli* according to procedures described previously [26]. Recombinant proteins were expressed in *E. coli* cells as fusion proteins with Glutathione-S-Transferase (GST) as the fusion partner, and purified to near homogeneity by affinity chromatography and treatment with thrombin protease, as described previously [27].

### Raising polyclonal antibodies against recombinant proteins in rabbits

Polyclonal antibodies were raised in rabbits against the purified and GST-free PE35, PPE68, ESXA, ESXB and ESXV recombinant proteins according to standard procedures [28]. In brief, purified proteins (50 µg/ml) were emulsified with an equal volume of incomplete Freund's adjuvant (IFA, SIGMA, USA) and injected intramuscularly in the right and left thigh. The rabbits were boosted twice with the same amount of protein at two weeks intervals. The animals were bled from the ear before each immunization and two weeks after the last immunization. The sera were stored frozen in aliquots at -20°C.

### Enzyme linked immunosorbent assay (ELISA)

ELISA was performed to detect antibodies in rabbit sera against full-length purified recombinant proteins and overlapping synthetic peptides corresponding to each protein using standard procedures [29]. In brief, wells of 96 well PolySorb plates (Nunc, USA) were coated with antigens/peptides (10 µg/ml), blocked with the blocking buffer, incubated with the primary antibody (rabbit 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 [30]. The color intensity was measured by determining the optical density (OD) at 405 nm. Antigen uncoated wells, and antigen coated wells but without adding primary antibody were used as negative controls. The results were expressed as E/C, which is defined as:

$E/C = OD \text{ in antigen coated wells with primary and secondary antibodies} / OD \text{ in antigen uncoated wells with primary and secondary antibodies}$ . The values of  $E/C > 2$  were considered positive.

## Western immunoblotting

Western immunoblotting was performed with rabbit sera and the purified recombinant proteins; sonicates, cell walls and culture filtrate of *M. tuberculosis*; and sonicates of *M. bovis* BCG, *M. avium* and *M. vaccae* using standard procedures [31]. In brief, the PE35, PPE68, ESXA, ESXB and ESXV recombinant proteins, mycobacterial sonicates, MT-CF and MT-CW were electrophoresed on sodium dodecyl sulfate-polyacrylamide gels and transferred to nitrocellulose membranes according to standard procedures [32,33]. The membranes were blocked, incubated with rabbit sera (diluted 1:100), followed by incubation with alkaline phosphatase conjugated anti-rabbit IgG and substrate, as described previously [28].

## Results

### Expression of PE35, PPE68, ESXA, ESXB, ESXV mRNA in *M. tuberculosis* and other mycobacteria

The natural expression of *pe35*, *ppe68*, *esxA*, *esxB* and *esxV* in *M. tuberculosis*, BCG, *M. avium*, and *M. vaccae* was studied at mRNA level by RT-PCR. The results showed that mRNA corresponding to *pe35*, *ppe68*, *esxA* and *esxB* were expressed in *M. tuberculosis* (Figure 1a), only *esxV* was expressed in BCG (Figure 1b) and mRNA for none of these genes were expressed in *M. avium* and *M. vaccae* (Figures 1c and 1d, respectively).

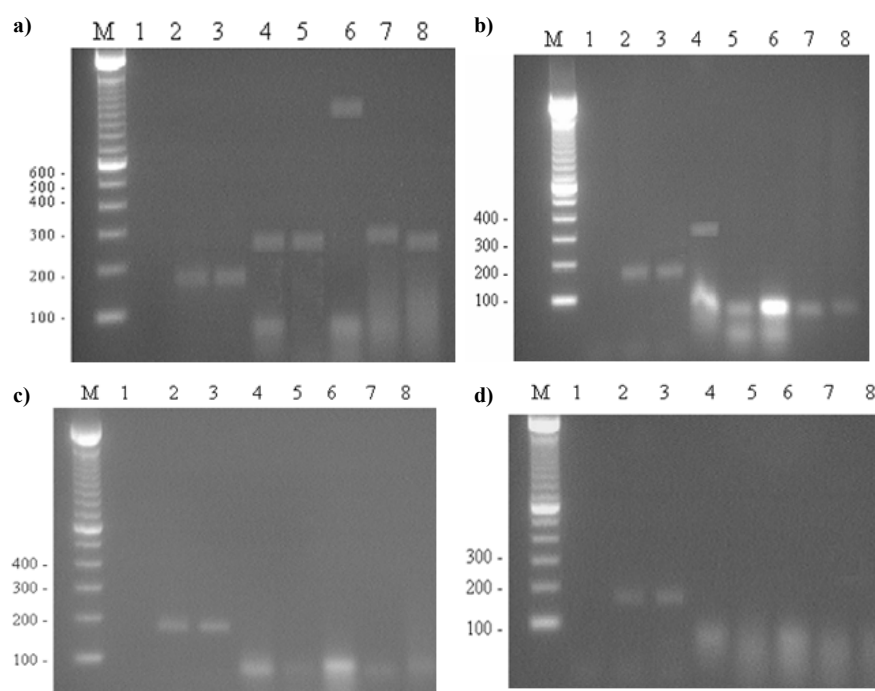
### Characterization of the reactivity of antigen-specific antibodies raised in rabbits

The GST-free purified recombinant PE35, PPE68, ESXA, ESXB and ESXV proteins were used to raise antigen-specific antibodies in

rabbits. Sera were obtained from all the rabbits before immunization and tested in Western immunoblots to confirm the absence of preexisting antibodies to the immunizing proteins (data not shown). Following immunization, sera were collected and tested for reactivity in Western immunoblots with pure proteins. The results showed that antibody reactivity was observed in the sera of all animals after the second booster, but only with the specific proteins that were used for immunization (Figure 2a, b, c, d, e), thus showing antigen-specificity of the antibodies. To determine the heterogeneity of response and the number of epitopes of recognized by rabbit antibodies, overlapping synthetic peptides corresponding to each protein were also tested with the sera using ELISA. The results showed that the antibodies, present in the sera of immunized rabbits, recognized several epitopes from different regions of each protein (Figure 3, results shown for PE35 and PPE68). These results suggest that each of the tested protein had several antibody-inducing epitopes scattered throughout the sequence.

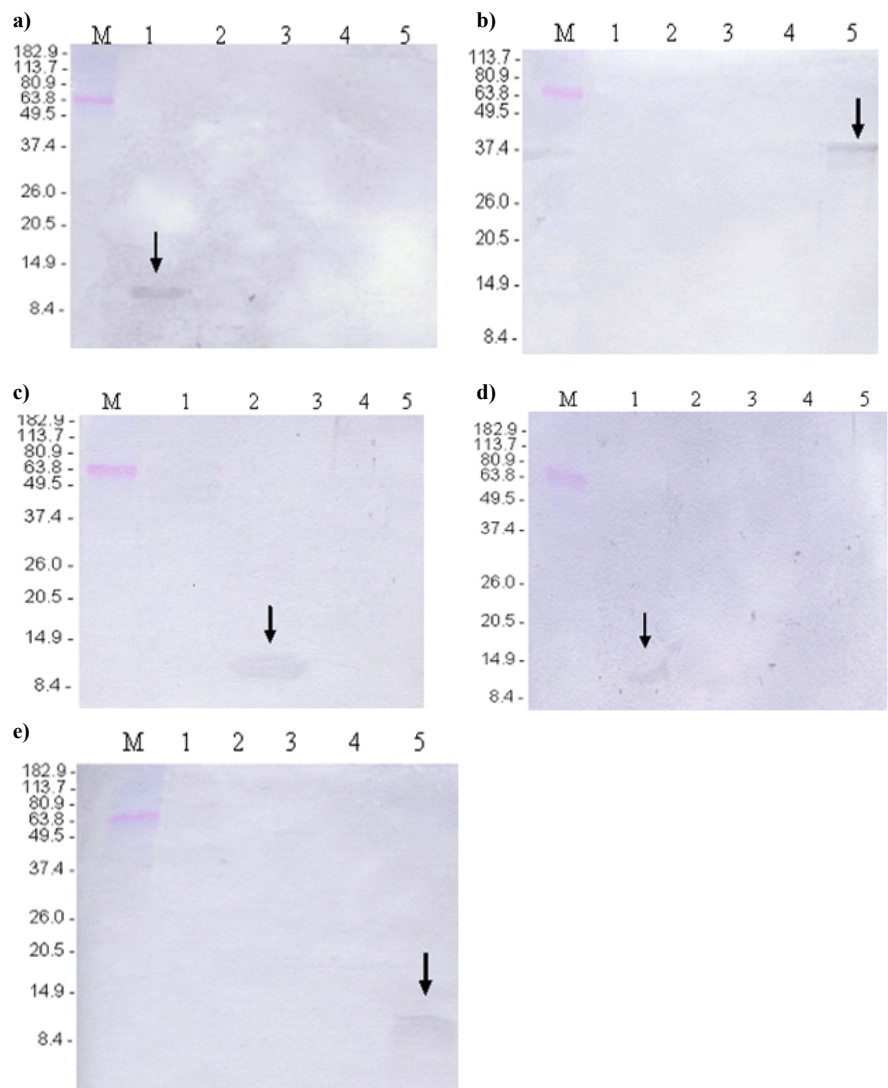
### Expression of PE35, PPE68, ESXA, ESXB, ESXV proteins in *M. tuberculosis* and other mycobacteria

The rabbit anti-sera containing antigen-specific polyclonal antibodies were used as probes in Western immunoblots to determine the natural expression of each protein in *M. tuberculosis* H<sub>37</sub>Rv, BCG, *M. avium*, and *M. vaccae* using sonicates of these mycobacteria. The results showed that proteins reactive with anti-PE35, -PPE68, -ESXA, -ESXB and -ESXV antibodies were present in *M. tuberculosis* H<sub>37</sub>Rv sonicate (Figure 4a, b, c, d, e); whereas, the BCG sonicate showed reactivity only with sera containing anti-ESXV antibodies (Figure 4e) and none of the sera showed antibody reactivity with sonicates of *M. avium* and *M. vaccae* (Figure 4a, b, c, d, e). The rabbit anti-sera against

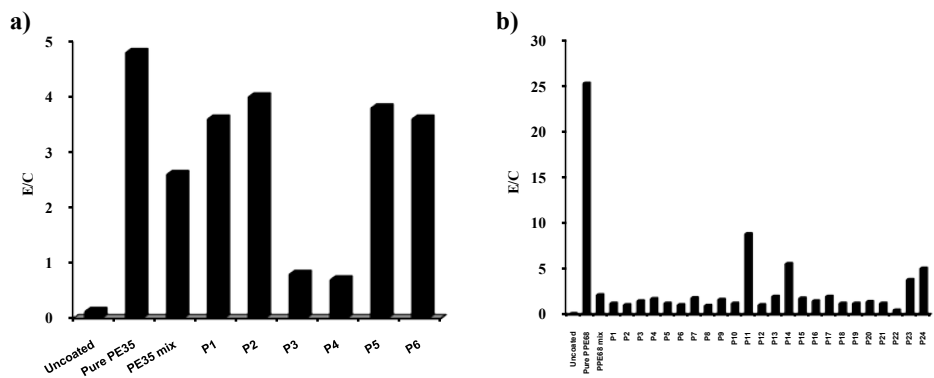


**Figure 1:** Agarose gel electrophoresis analysis of PCR amplified DNA using sequence-specific primers and RNA, genomic DNA and cDNA synthesized from RNA of *M. tuberculosis* (a), BCG (b), *M. avium* (c), *M. vaccae* (d). M: 100 bp DNA ladder, Lane 1: RNA amplified with MD1/MD2 primers (No product). Lane 2: Genomic DNA amplified with MD1/MD2 primers (162 bp DNA product). Lane 3: cDNA amplified with MD1/MD2 primers (162 bp DNA product), Lane 4: cDNA amplified with ESXV primers (301 bp DNA product) Lane 5: cDNA amplified with PE35 primers (316 bp DNA product), Lane 6: cDNA amplified with PPE68 primers (1123 bp DNA product), Lane 7: cDNA amplified with ESXB primers (307 bp DNA product), Lane 8: cDNA amplified with ESXA primers (319 bp DNA product).

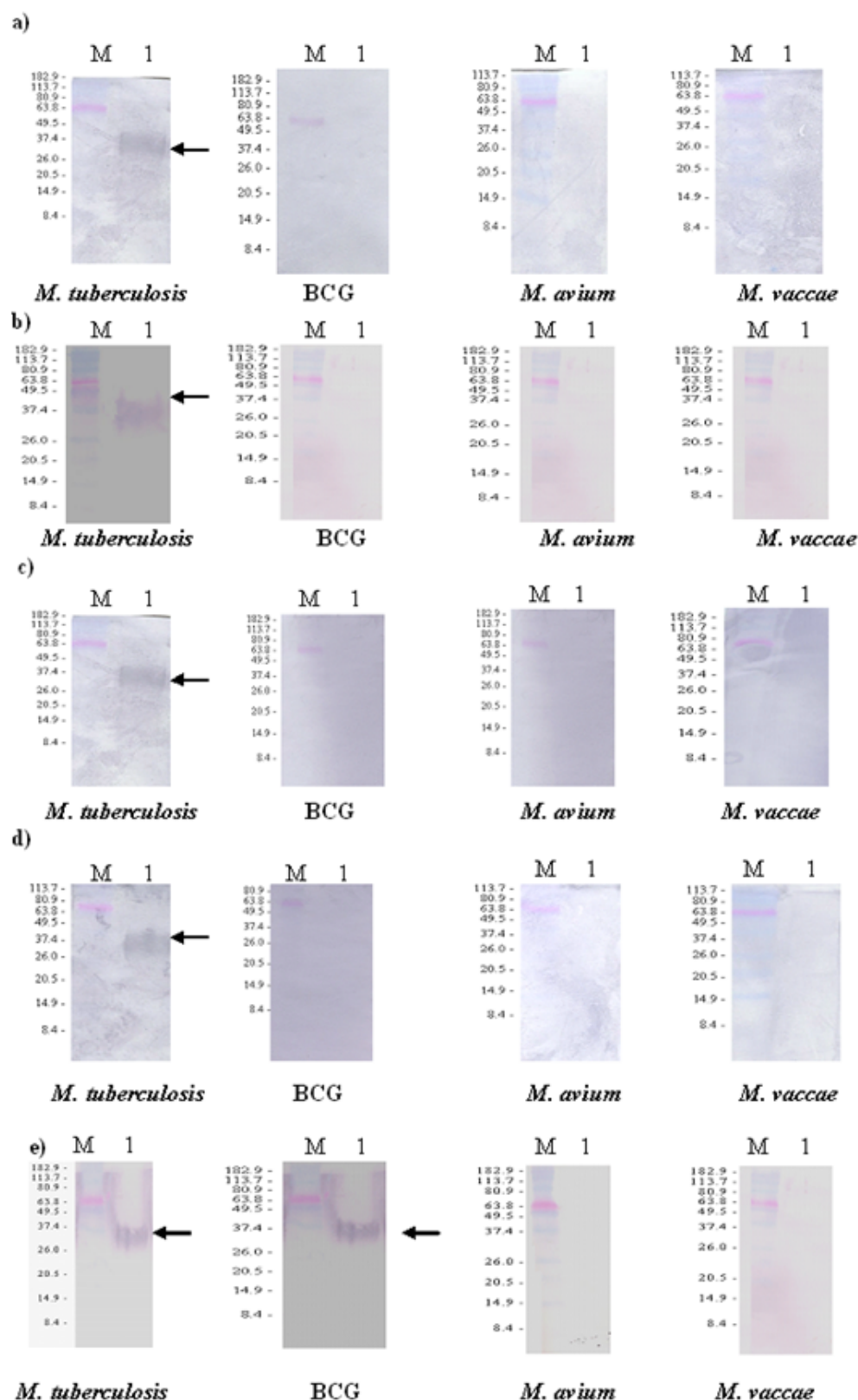




**Figure 2:** Western immunoblots with pure recombinant proteins and rabbit sera obtained after second booster from animals immunized with pure PE35 (panel a), PPE68 (panel b), ESXA (panel c), ESXB (panel d) and ESXV (panel e). M: Prestained low molecular weight marker. The arrow in each panel shows the band for antibody reactivity with the specific protein that was used for immunization, i.e. panel a, lane 1: PE35; panel b, lane 5: PPE68; panel c, lane 2: ESXA, panel d, lane 1: ESXB, panel e, lane 5: ESXV. The other lanes in each panel were loaded with other proteins.



**Figure 3:** ELISA reactivity of antibodies present in rabbit sera from animals immunized with pure recombinant PE35 (panel a) and PPE68 (panel b) with pure proteins, peptide mixtures and individual peptides of PE35 and PPE68, respectively.



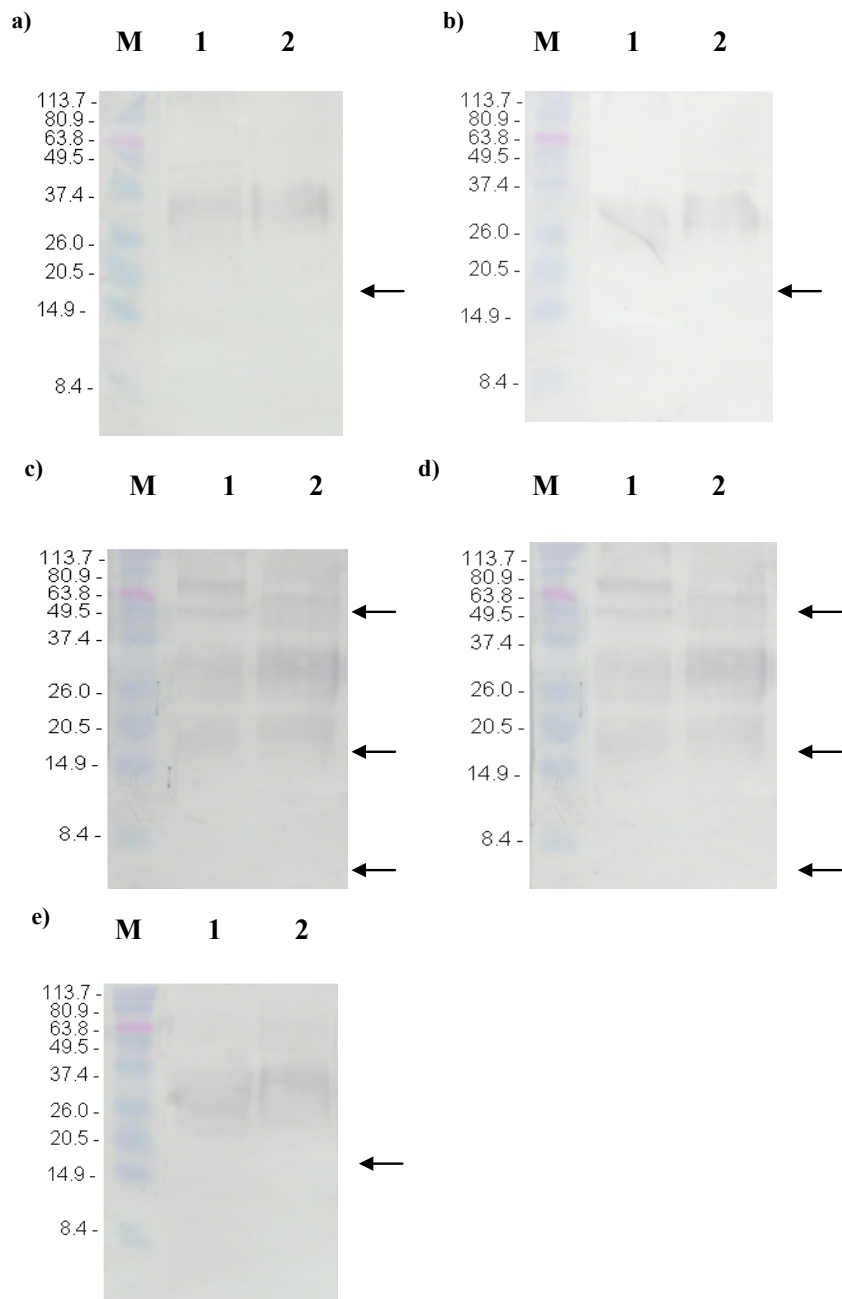
**Figure 4:** Western immunoblot analysis of proteins in mycobacterial sonicates with sera of rabbits immunized with pure recombinant PE35 (a), PPE68 (b), ESXA (c), ESXB (d), and ESXV (e). M: Prestained low molecular weight marker. Lane 1: Mycobacterial sonicate from the species indicated. The arrows indicate the protein band reactive with antibodies.

all proteins also showed antibody reactivities with the culture filtrate and cell wall preparations of *M. tuberculosis* (Figure 5). These results suggest that PE35, PPE68, ESXA, ESXB and ESXV proteins were expressed in *M. tuberculosis* and ESXV protein in BCG. However, the strongest reactivities observed with all the preparations of *M. tuberculosis* with anti-PE35, -ESXA, -ESXB and -ESXV sera and of BCG sonicate with anti-ESXV sera were at the molecular mass of about 35 kDa instead of about 10 kDa observed with pure proteins. These observations suggest that the expressed proteins were multimerized or

associated with other small molecular weight proteins expressed in the bacilli.

**Discussion**

The antigens encoded by *M. tuberculosis*-specific RDs may be useful in the specific diagnosis of TB and development of new vaccines [7,8]. However, it is required that antigens selected for these applications should only be expressed in *M. tuberculosis* and not in other mycobacteria, particularly in BCG vaccine and environmental



**Figure 5:** Western immunoblot analysis of proteins present in the culture filtrate (MT-CF) and cell wall (MT-CW) preparations of *M. tuberculosis* after probing with sera obtained from rabbits immunized with pure recombinant PE35 (a), PPE68 (b), ESXA (c), ESXB (d), and ESXV (e). M: Prestained low molecular weight marker. Lane 1: MT-CF, Lane 2: MT-CW. The arrows indicate regions of antibody reactivity.

mycobacteria. Previous studies have identified PE35, PPE68, ESXA, ESXB of RD1 and ESXV of RD9 as major antigens recognized by humans [15,34,35]. This study was conducted to determine the expression of their genes in *M. tuberculosis* as well as the specificity of expression. Towards this end, their expression in *M. tuberculosis*, BCG and two species of environmental mycobacteria (*M. avium* and *M. vaccae*) was first studied at the mRNA level by RT-PCR. The results showed that PE35, PPE68, ESXA and ESXB mRNA were expressed in *M. tuberculosis* but not in BCG, *M. vaccae* and *M. avium*. On the other hand, ESXV mRNA was expressed in *M. tuberculosis* as well as in BCG but not in *M. avium* and *M. vaccae*. A previous study by Amoudy et al. also showed the expression of RD1 PE35, PPE68, ESXA and ESXB mRNA in *M. tuberculosis* [23]. The expression of PPE68 and ESXA has also been shown by other workers in *M. tuberculosis* but not in BCG [34,35]. However, to our knowledge, this is the first study to demonstrate the expression of all these genes at mRNA level in *M. tuberculosis*, only ESXV in *M. bovis* BCG and none of them in the environmental mycobacterial species of *M. avium* and *M. vaccae*.

The expression of *pe35*, *ppe68*, *esxA*, *esxB* and *esxV* genes was studied at the protein level by using polyclonal antibodies raised in rabbits against all of the five pure recombinant proteins. The specificity of the antibodies was confirmed by Western immunoblot analysis, which demonstrated that sera from pre-immunized rabbits did not have antibodies to any of these proteins, and the sera from immunized rabbits had antibodies reactive with the immunizing proteins only. These results suggest that the rabbits used were not exposed to *M. tuberculosis* and the epitopes of a given protein recognized by antibodies were not crossreactive with other proteins. When sera containing these antigen-specific antibodies were tested for expression of the respective proteins in *M. tuberculosis* and other mycobacteria (BCG, *M. vaccae* and *M. avium*), it was found that the sera containing antibodies against all of the proteins reacted with the sonicates of *M. tuberculosis* H<sub>37</sub>Rv, only sera containing anti-ESXV antibodies reacted with BCG sonicate and none of the sera reacted with *M. avium* and *M. vaccae* sonicates. By using synthetic peptides in ELISA, we found that the antibodies in rabbit sera recognized several epitopes in these proteins. The lack of reactivity of these sera with BCG (in case of PE35, PPE68, ESXA and ESXB) and *M. avium* and *M. vaccae* suggest that, in addition to lacking the respective full-length genes as demonstrated by RT-PCR, these mycobacteria do not express proteins with crossreactive epitopes.

The results of RT-CR and Western immunoblots confirm the expression of ESXV mRNA and proteins in *M. tuberculosis* and BCG, while PE35, PPE68, ESXA and ESXB mRNA and proteins are expressed in *M. tuberculosis* only. The expression of PE35, PPE68, ESXA and ESXB in *M. tuberculosis* but not in BCG and other mycobacteria was expected because the respective genes are present in RD1 genomic region, which is deleted in BCG and many other mycobacteria [9,36]. However, the expression of ESXV in *M. tuberculosis* as well as in BCG was not expected because this gene is present in RD9 segment of *M. tuberculosis*, which is also deleted in BCG [9,36]. To resolve this discrepancy, a protein BLAST search was performed using the sequence of ESXV by accessing Tuberculist world-wide web server (<http://genolist.pasteur.fr/TubercuList/>) to determine the presence of a homolog in *M. tuberculosis* which may be identical/similar to ESXV. The search showed that ESXV is 100% identical with another protein of ESX-family, i.e. ESXI [16]. The gene predicted to encode ESXI is present in the genome region of *M. tuberculosis* which is shared with BCG. Thus, two identical copies of the same gene occur in *M. tuberculosis*, one copy in RD9 as ESXV, which is deleted in BCG, and the other copy outside RD9 as ESXI, which is conserved in BCG.

Further experiments were performed to determine the sub-cellular localization of the proteins included in this study by testing MT-CF and MT-CW in Western immunoblots using rabbit sera containing antigen-specific antibodies. The results of these experiments show that antigen-specific antibodies present in rabbit sera reacted with proteins present in the both MT-CF and MT-CW preparations of *M. tuberculosis*. These results suggest that all of these proteins were either actively secreted or released from the growing organisms in 14 days culture period, but they were also entrapped within the cell wall structure during in vitro growth of *M. tuberculosis*. Consistent with our study, association of PPE68 with mycobacterial cells and cell walls has been shown by demonstrating that this protein was mainly detected in the lysate and cell wall fractions and smaller amounts were present in the membrane fraction [34,35]. Furthermore the presence of ESXA and ESXB has been reported in culture filtrates, cell walls, cytosol and cell membrane of *M. tuberculosis* [36-38].

The presence of all of these proteins in the cell walls would suggest that live mycobacteria may not be must to study the in vivo immunogenicity of RD1 and RD9 proteins included in the study. In line with this suggestion, we have recently shown that immunization of guinea-pigs with killed *M. tuberculosis* induced delayed type hypersensitivity responses to all these proteins [16]. On the other hand, live mycobacteria would have been essential for immunogenicity studies if these proteins were only present in culture filtrate but not in cytoplasmic or cell wall preparations.

In conclusion, our study demonstrates that the five major antigenic proteins encoded by RD1, i.e. PE35, PPE68, ESXA, ESXB, and RD9, i.e. ESXV, are expressed in *M. tuberculosis* at both mRNA and protein levels, whereas only ESXV is expressed in *M. bovis* BCG and none of these proteins are expressed in the environmental mycobacterial species of *M. avium* and *M. vaccae*. As these proteins are major antigens encoded by RD1 and RD9 of *M. tuberculosis* [39-43], they could be useful in tuberculosis control both in relation to specific diagnosis and development of new vaccines.

#### Acknowledgements

This study was supported by the College of Graduate Studies and Research Administration, Kuwait University, Kuwait grant YM01/03. We are grateful to Mr. Sunny Ojoko and Dr. Severino Gabato for help in providing, caring and bleeding rabbits.

#### Ethical Approval

Rabbits were immunized and handled according to established IACUC-approved protocols at Kuwait University, Kuwait.

#### References

1. Global tuberculosis control: epidemiology, strategy, financing. WHO Report 2009 WHO/HTM/TB/2009.411.
2. Lönnroth K, Raviglione M (2008) Global epidemiology of tuberculosis: prospects for control. *Semin Respir Crit Care Med* 29: 481-491.
3. Hill PC, Brookes RH, Adetifa IM, Annette Fox, Dolly Jackson-Sillah, et al. (2006) Comparison of enzyme-linked immunospot assay and tuberculin skin test in healthy children exposed to Mycobacterium tuberculosis. *Pediatrics* 117: 1542-1548.
4. Calmette A, Guérin C, Negre L, Boquet A. Prémunition des nouveaux-nés contre la tuberculose par le vaccin BCG, 1921-1926. *Ann Ins. Pasteur (Paris)* 40: 89-133.
5. Crampin AC, Glynn JR, Fine PE (2009) What has Karonga taught us? Tuberculosis studied over three decades. *Int J Tuberc Lung Dis* 13: 153-164.
6. Hesselting AC, Caldwell J, Cotton MF, Eley BS, Jaspan HB et al. (2009) BCG vaccination in South African HIV-exposed infants-risks and benefits. *S Afr Med J* 99: 88-91.



7. Mustafa AS (2009) Vaccine potential of Mycobacterium tuberculosis-specific genomic regions: in vitro studies in humans. *Expert Rev Vaccines* 8: 1309-1312.
8. Arend SM, Franken WP, Aggerbeck H, Prins C, van Dissel JT, et al. (2008) Double-blind randomized Phase I study comparing rESAT-6 to tuberculin as skin test reagent in the diagnosis of tuberculosis infection. *Tuberculosis (Edinb)* 88: 249-261.
9. Behr MA, Wilson MA, Gill WP, Salamon H, Schoolnik GK, et al. (1999) Comparative genomics of BCG vaccines by whole-genome DNA microarray. *Science* 284: 1520-1523.
10. Al-Attayah R, Mustafa AS (2008) Characterization of human cellular immune responses to novel Mycobacterium tuberculosis antigens encoded by genomic regions absent in Mycobacterium bovis BCG. *Infect Immun* 76: 4190-4198.
11. Mustafa AS, Al-Attayah R (2009) Identification of Mycobacterium tuberculosis-specific genomic regions encoding antigens inducing protective cellular immune responses. *Indian J Exp Biol* 47: 498-504.
12. Mustafa AS, Al-Attayah R, Hanif SNM, Shaban FA (2008) 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* 15: 916-924.
13. Hanif SNM, El-Shamy AM, Al-Attayah R, Mustafa AS (2008) Whole blood assays to identify Th1 cell antigens and peptides encoded by Mycobacterium tuberculosis-specific RD1 genes. *Med Princ Pract* 17: 244-249.
14. Mukherjee P, Dutta M, Datta P, Pradhan, Kundu, et al. (2007) The RD1-encoded antigen Rv3872 of Mycobacterium tuberculosis as a potential candidate for serodiagnosis of tuberculosis. *Clin Microbiol Infect* 13: 146-152.
15. Mustafa AS, Al-Attayah R (2004) Mycobacterium tuberculosis antigens and peptides as new vaccine candidates and immunodiagnostic reagents against tuberculosis. *Kuwait Med J* 36: 171-176.
16. Hanif SNM, Al-Attayah R, Mustafa AS (2010) Species-specific antigenic proteins of Mycobacterium tuberculosis as tested by DTH response in guinea pigs. *Int J Tub Lung Dis* 14: 489-494.
17. Cole ST, Brosch R, Parkhill J, Garnier T, Churcher C, et al. (1998) Deciphering the biology of Mycobacterium tuberculosis from the complete genome sequence. *Nature* 393: 537-544.
18. Al-Attayah R, Mustafa AS (2009) Mycobacterial antigen-induced T helper type 1 (Th1) and Th2 reactivity of peripheral blood mononuclear cells from diabetic and non-diabetic tuberculosis patients and Mycobacterium bovis bacilli Calmette-Guerin (BCG)-vaccinated healthy subjects. *Clin Exp Immunol* 158: 64-73.
19. Mustafa AS, Cockle PJ, Shaban F, Hewinson RG, Vordermeier HM (2002) Immunogenicity of Mycobacterium tuberculosis RD1 region gene products in infected cattle. *Clin Exp Immunol* 130: 37-42.
20. Mustafa AS (2009) Th1-cell reactivity and HLA-DR binding prediction for promiscuous recognition of MPT63 (Rv1926c), a major secreted protein of Mycobacterium tuberculosis. *Scand J Immunol* 69: 213-222.
21. Mustafa AS, Abal AT, Shaban F, El-Shamy AM, Amoudy HA (2005) HLA-DR binding prediction and experimental evaluation of mycolyltransferase (Ag85B), a major secreted antigen of Mycobacterium tuberculosis. *Med Princ Pract* 14: 140-146.
22. Mustafa AS, Shaban FA (2006) Propred analysis and experimental evaluation of promiscuous Th1 cell epitopes of three major secreted antigens of Mycobacterium tuberculosis. *Tuberculosis (Edinb)* 86: 115-124.
23. Amoudy HA, Al-Turab MB, Mustafa AS (2006) Identification of transcriptionally active open reading frames within the RD1 genomic segment of Mycobacterium tuberculosis. *Med Princ Pract* 15: 137-144.
24. Qasem JA, Mustafa AS, Khan ZU (2008) Legionella in clinical specimens and hospital water supply facilities: molecular detection and genotyping of the isolates. *Med Princ Pract* 17: 49-55.
25. Amoudy HA, Mustafa AS (2008) Amplification of Six Putative RD1 Genes of Mycobacterium tuberculosis for Cloning and Expression in Escherichia coli and Purification of Expressed Proteins. *Med Princ Pract* 17: 378-384.
26. Ahmad S, Ali MM, Mustafa AS (2003) Construction of a modified vector for efficient purification of recombinant Mycobacterium tuberculosis proteins expressed in Escherichia coli. *Protein Expr Purif* 29: 167-175.
27. Ahmad S, El-Shazly S, Mustafa AS, Al-Attayah R (2004) Mammalian cell-entry proteins encoded by the mce3 operon of Mycobacterium tuberculosis are expressed during natural infection in humans. *Scand J Immunol* 60: 382-391.
28. Ahmad S, El-Shazly S, Mustafa AS, Al-Attayah R (2005) The six mammalian cell-entry proteins (Mce3A-F) are expressed during in vitro growth of Mycobacterium tuberculosis. *Scand J Immunol* 62: 16-24.
29. Amoudy HA, Al-Asmer ABH, Abul AT, Mustafa AS (1997) Evaluation of complex and defined antigens of Mycobacterium tuberculosis in an IgG specific ELISA for the diagnosis of tuberculosis. *Med Princ Pract* 6: 103-109.
30. El-Shazly S, Mustafa AS, Ahmad S, Al-Attayah R (2007) Utility of three mammalian cell entry proteins of Mycobacterium tuberculosis in serodiagnosis of tuberculosis. *Int J Tuberc Lung Dis* 11: 676-682.
31. Amoudy HA, Ahmad S, Thole JE, Mustafa AS (2007) Demonstration of in vivo expression of a hypothetical open reading frame (ORF-14) encoded by the RD1 region of Mycobacterium tuberculosis. *Scand J Immunol* 66: 422-425.
32. Ahmad S, Amoudy HA, Thole JE, Young DB, Mustafa AS (1999) Identification of a novel protein antigen encoded by a Mycobacterium tuberculosis-specific RD1 region gene. *Scand J Immunol* 49: 515-522.
33. Ahmad S, Akbar PK, Wiker HG, Harboe M, Mustafa AS (1999) Cloning, expression and immunological reactivity of two mammalian cell entry proteins encoded by the mce1 operon of Mycobacterium tuberculosis. *Scand J Immunol* 50: 510-518.
34. Okkels LM, Brock I, Follmann F, Else Marie Agger, Sandra M. Arend, et al. (2003) PPE protein (Rv3873) from DNA segment RD1 of Mycobacterium tuberculosis: strong recognition of both specific T-cell epitopes and epitopes conserved within the PPE family. *Infect Immun* 71: 6116-6123.
35. Demangel C, Brodin P, Cockle PJ, Roland Brosch, Laleh Majlessi, et al. (2004) Cell envelope protein PPE68 contributes to Mycobacterium tuberculosis RD1 immunogenicity independently of a 10-kilodalton culture filtrate protein and ESAT-6. *Infect Immun* 72: 2170-2176.
36. Harboe M, Oettinger T, Wiker HG, Rosenkrands I, Andersen P (1996) Evidence for occurrence of the ESAT-6 protein in Mycobacterium tuberculosis and virulent Mycobacterium bovis and for its absence in Mycobacterium bovis BCG. *Infect. Immun* 64: 16-22.
37. Spencer JS, Kim HJ, Marques AM, Mercedes Gonzalez-Juarerro, Monica CBS, et al. (2004) Comparative analysis of B- and T-cell epitopes of Mycobacterium leprae and Mycobacterium tuberculosis culture filtrate protein 10. *Infect. Immun* 72: 3161-3170.
38. Sorensen AL, Nagai S, Houen G, Andersen P, Andersen AB (1995) Purification and characterization of a lowmolecular-mass T-cell antigen secreted by Mycobacterium tuberculosis. *Infect Immun* 63: 1710-1717.
39. Hanif SN, Al-Attayah R, Mustafa AS (2010) 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* 71: 353-361.
40. Mustafa AS (2010) Cell mediated immunity assays identify proteins of diagnostic and vaccine potential from genomic regions of difference of Mycobacterium tuberculosis. *Kuwait Med J* 42: 98-105.
41. Hanif SN, Al-Attayah R, Mustafa AS (2011) Cellular Immune Responses in Mice Induced by M. tuberculosis PE35-DNA Vaccine Construct. *Scand J Immunol* 74: 554-560.
42. Hanif SN, Al-Attayah R, Mustafa AS (2010) DNA vaccine constructs expressing Mycobacterium tuberculosis-specific genes induce immune responses. *Scand J Immunol* 72: 408-415.
43. Mustafa AS, Al-Saidi F, El-Shamy AS, Al-Attayah R (2011) Cytokines in response to proteins predicted in genomic regions of difference of Mycobacterium tuberculosis. *Microbiol Immunol* 55: 267-278.