Medical Principles and Practice

Med Princ Pract 2013;22(suppl 1):43–51
DOI: 10.1159/000354206

Received: April 16, 2013
Accepted: July 8, 2013
Published online: August 31, 2013

In silico Analysis and Experimental Validation of *Mycobacterium tuberculosis*-Specific Proteins and Peptides of *Mycobacterium tuberculosis* for Immunological Diagnosis and Vaccine Development

Abu Salim Mustafa

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

Key Words
In silico analysis · *Mycobacterium tuberculosis* · Peptides · Proteins · Diagnosis · Vaccine

Abstract
Comparative analyses of the *Mycobacterium tuberculosis* genome with the genomes of other mycobacteria have led to the identification of several genomic regions of difference (RDs) between *M. tuberculosis* and *M. bovis* BCG. The identification of immunodominant and HLA-promiscuous antigens and peptides encoded by these RDs could be useful for diagnosis and the development of new vaccines against tuberculosis. The analysis of RD proteins and peptides by in silico methods (using computational programs to predict major and HLA-promiscuous antigenic proteins and peptides) and experimental validations (using peripheral blood mononuclear cells and sera from tuberculosis patients and BCG-vaccinated healthy subjects to assess antigen-specific cellular and humoral immune responses in vitro) identified several major antigens and peptides. To evaluate the in vivo potentials, the genes of immunodominant antigens were cloned and expressed in DNA vaccine vectors. Immunizations of experimental animals with the recombinant constructs induced antigen-specific cellular responses. Further experiments showed that each of these proteins had several T and B cell epitopes scattered throughout their sequence, which confirmed their strong immunogenicity. In conclusion, the bioinformatics-based in silico identification of promiscuous antigens and peptides of *M. tuberculosis* is a useful approach to identify new candidates important for diagnosis and vaccine applications.
© 2013 S. Karger AG, Basel

Introduction

Tuberculosis (TB) is a major infectious disease problem of worldwide prevalence and ranks among the top 10 causes of global mortality. The most recent estimates from the WHO suggest that there are 8.7 million new cases of TB (range 8.3–9.0 million) globally, equivalent to 125 cases per 100,000 individuals, and 1.4 million people died from TB in 2011, including almost one million deaths among HIV-negative individuals and 430,000 among people who were coinfected with HIV [1]. Furthermore, it is estimated that one third of the world's population is latently infected with *Mycobacterium tuberculosis* and about 10% of these people will develop active disease in their lifetime. In spite of worldwide efforts to control TB, the global burden of the disease is worsening in the poor developing countries of Asia and Africa. This is due to many reasons, including wars and immigration, poverty and malnutrition, HIV-TB coinfection, and the increasing problem of multidrug-resistant and excessive drug-resistant TB, etc. [1]. The worldwide control of TB

KARGER

E-Mail karger@karger.com
www.karger.com/mpp

© 2013 S. Karger AG, Basel
1011–7571/13/0227–0043$38.00/0

Karger Open access

This is an Open Access article licensed under the terms of the Creative Commons Attribution-NonCommercial 3.0 Unported license (CC BY-NC) (www.karger.com/OA-license), applicable to the online version of the article only. Distribution permitted for non-commercial purposes only.

Abu Salim Mustafa
Department of Microbiology
Faculty of Medicine, Kuwait University
PO Box 24923, Safat 13110 (Kuwait)
E-Mail abusalim@hsc.edu.kw

requires an effective control and eradication strategy using cost-effective methods/reagents for specific diagnosis and prophylactic and/or immunotherapeutic vaccine(s) that can be given safely [2, 3]. In particular, effective vaccines are considered the best weapons to fight against infectious diseases [2].

Tuberculin Skin Test for the Diagnosis of TB

Currently, the tuberculin skin test (TST) is the only test available globally for the in vivo immunological diagnosis of TB. The antigenic preparation in TST is the purified protein derivative (PPD) of *M. tuberculosis,* which is a crude mixture of molecules present in the culture filtrate of in vitro grown *M. tuberculosis.* Hence, the PPD contains substances that are *M. tuberculosis* specific as well cross-reactive with other mycobacteria. Although it is a simple and cost-effective test, TST results must be interpreted carefully because a negative test does not rule out a diagnosis of TB but may reflect the presence of nonresponsiveness due to the immunocompromised state of the patients or incorrect test procedures [4]. In addition, a positive TST cannot distinguish between active disease, latent infection with *M. tuberculosis,* BCG vaccination, or cross-sensitization by environmental mycobacteria [4]. Thus, the TST has poor diagnostic value, especially in geographic areas and countries where BCG is routinely used, the prevalence of TB is low, or the environmental burden of nontuberculous environmental mycobacteria is high [3–6]. Therefore, a TB-specific skin test requires the development of new tuberculin(s) consisting of antigens specific for *M. tuberculosis.*

M. bovis BCG as a Vaccine against TB

The currently available vaccine against TB is BCG, which is a live attenuated strain of *M. bovis.* Although it has been widely used to vaccinate against TB for about 90 years, BCG is the most controversial vaccine being used in humans. This is because BCG has failed to protect against TB in different parts of the world, especially in adults with pulmonary TB [3, 7]. The variations in protection have ranged from nil (e.g. in India and Malawi) to 80% (e.g. in the UK) against pulmonary TB in adults [3, 8]. Furthermore, BCG is not suitable for vaccination of immunocompromised individuals, particularly HIV/AIDS patients, due to the fear of causing disease in such individuals [9]. Moreover, because BCG vaccination induces a positive skin test response to PPD, it becomes difficult to use TST for diagnostic or epidemiological investigations in BCG-vaccinated populations [3]. Therefore, the development of new vaccines based on *M. tuberculosis*-specific antigens is urgently needed.

Comparative Genomics to Identify M. tuberculosis-Specific Genomic Regions Deleted/Absent in M. bovis BCG and Approaches to Obtain Regions of Difference Proteins or Peptide Equivalents for Immunological Evaluation

The identification of *M. tuberculosis*-specific antigens has been facilitated by the relatively recent advances in genomics of mycobacteria, leading to the sequencing of complete genomes of various mycobacterial species, including *M. tuberculosis, M. bovis,* and BCG, etc. Comparative analyses of mycobacterial genomes have identified 16 genomic regions of *M. tuberculosis,* which are deleted/absent in one or more strains of BCG [10, 11]. Among these regions of difference (RDs), 11 RDs (RD1, RD4 to RD7, RD9 to RD13, and RD15) of *M. tuberculosis* H37Rv are absent in all BCG substrains currently used as vaccines to protect against TB in different parts of the world (table 1). In silico analysis suggested that these RDs have 86 open reading frames (ORFs) capable of encoding equal numbers of proteins (table 1), [10]. Further studies have suggested that in silico predictions of RD ORFs could be relevant to protein expression in *M. tuberculosis* because the expression of all of the predicted RD1 genes, at the mRNA level, was confirmed in *M. tuberculosis* using reverse-transcriptase PCR assays [12, 13]. Attempts have been made to obtain RD proteins by using recombinant methods of DNA cloning and expression, followed by purification of recombinantly expressed proteins, or their equivalents using overlapping synthetic peptides covering the sequence of each protein [14–17]. As described below, these recombinant antigens and synthetic peptides have been tested in antibody and cellular assays to identify the candidate RD proteins/peptides suitable for diagnosis and vaccine development.

Purified RD Proteins and Synthetic Peptides for Immunological Reactivity

To characterize RD proteins for immunological reactivity, it is essential that these proteins be obtained in a purified form, free from other cellular proteins of *M. tu-*

Table 1. Some features of 11 RDs of *M. tuberculosis* deleted/absent in all *M. bovis* BCG vaccine strains

RD		Predicted ORFs in each RD		Synthetic peptides to cover the ORFs of each RD, n
designation	size, kb	n	designation	
RD1	9.5	9	Rv3871-Rv3879	220
RD4	1.9	3	Rv0221-Rv0223c	80
RD5	2.8	5	Rv3117-Rv3121	72
RD6	12.8	11	Rv1506c-Rv1516c	236
RD7	9.0	8	Rv2346c-Rv2353c	167
RD9	5.9	7	Rv3617-Rv3623	108
RD10	3.0	3	Rv1255c-Rv1257c	71
RD11	4.9	5	Rv3425-Rv3429	84
RD12	2.0	4	Rv2072c-Rv2075c	83
RD13	11.0	16	Rv2645-Rv2660c	225
RD15	12.7	15	Rv1963c-Rv1977	302
Total peptides				1,648

berculosis. The natural expression of all putative/predicted RD proteins in *M. tuberculosis* could not be determined due to the lack of reagents which are required to obtain purified proteins from *M. tuberculosis* cultures. On the other hand, the DNA sequences of all of the putative/predicted RD proteins were known from the genome sequence [10]; therefore, attempts were made to express them in *Escherichia coli* cells and purify the recombinant mycobacterial proteins in quantities sufficient for immunological assays [18–25]. However, the production of mycobacterial proteins in general and proteins of large size in particular has been quite difficult in *E. coli* for various reasons, including difficulties in amplification of target genes from genomic DNA of *M. tuberculosis,* degradation of the expressed proteins by *E. coli* proteases, and problems in purification of mycobacterial proteins from *E. coli* components [18–24].

To overcome the problems associated with recombinant protein expression and purification, pools of synthetic peptides covering the coding sequence of mycobacterial proteins have been used to faithfully replace full-length proteins in immunological assays [26–35]. Therefore, to test 86 ORFs of 11 RDs of *M. tuberculosis* in immunological assays, a total of 1,648 synthetic peptides were synthesized [36–40], (table 1). All of the peptides were 25-mers and overlapped with the neighboring peptides of the same ORF by 10 residues [41–45]. The purpose of the 10-residue overlap was to greatly reduce the probability of missing the epitopes of individual ORFs, because mycobacterial epitopes recognized in immunological assays are usually <10 aa in length [46].

Identification of Major RD-Encoded Proteins/Peptides Reactive in Antibody Assays

A total of 775 synthetic peptides covering the sequence of 39 ORF proteins encoded by genes predicted in 5 RDs of *M. tuberculosis,* i.e. RD1, RD4, RD5, RD6, and RD7, were tested by enzyme-linked immunosorbent assays for antibody reactivity with sera from HIV-negative pulmonary TB patients (n = 100) and *M. bovis* BCG-vaccinated healthy subjects (n = 100). The results identified 4 immunodominant peptides reactive with TB sera, i.e. aa 346–370 of RD1ORF Rv3876, aa 241–265 of RD6ORF Rv1508c, aa 136–160 of RD6ORF Rv1510, and aa 325–336 of RD6ORF Rv1516c [47]. However, only 3 of them, i.e. aa 346–370 of Rv3876, aa 241–265 of Rv1508c, and aa 325–336 of Rv1516c, had significantly stronger antibody reactivity with sera from TB patients versus healthy subjects ($p < 0.05$), and significantly higher positivity with TB sera (% positives = 66–93%) versus sera from healthy subjects (% positives = 10–28%) [47]. In silico analysis for antibody epitopes using an ABCPred server showed that each of the peptides had multiple epitopes for recognition by antibodies. To verify these predictions, anti-peptide antibodies were raised in rabbits after immunization with pools of 11 peptides corresponding to each protein. The results showed that these peptides could induce production of antibodies in vivo. Probing of culture filtrate and whole cell lysates of *M. tuberculosis* with antipeptide antibodies suggested the natural expression of Rv3876, Rv1508c, and Rv1516c in whole cell lysates of *M. tuberculosis.* These results suggest that the immunodominant RD peptides with serodiagnostic potential are naturally

Table 2. Summary of the names of the RD1 genes, the ORF annotation, and the proteins

Gene name	ORF annotation	Protein name
Rv3871	Rv3871	Rv3871
pe35	Rv3872	PE35
ppe68	Rv3873	PPE68
esxb/cfp10	Rv3874	ESXB/CFP10
esxa/esat6	Rv3875	ESXA/ESAT-6
Rv3876	Rv3876	Rv3876
Rv3877	Rv3877	Rv3877
Rv3878	Rv3878	Rv3878
Rv3879c	Rv3879	Rv3879

expressed in *M. tuberculosis* [47]. A number of other studies also suggest the diagnostic potential of RD proteins and peptides based on detection of antibodies in sera of TB patients [48–50].

In silico Analysis of RD Proteins for T Cell Epitopes

The presentation of antigens to T cells requires their processing into small peptides by antigen-presenting cells and association with major histocompatibility complex (MHC) molecules. The length of T cell epitopes usually ranges from 8 to 10 aa, which could easily be synthesized using standard chemistries to overcome the problems associated with obtaining full-length recombinant proteins. The use of synthetic peptides in TB has been successfully demonstrated for diagnostic applications in humans and cattle [51, 52], and as a protective vaccine in mice [53]. In most studies, overlapping synthetic peptides covering the entire sequence of a protein have been used to identify the peptides recognized by T cells [54–57]. However, usually a fraction of peptides of large size proteins are recognized by T cells [54–60], and therefore the overlapping peptide approach becomes quite costly and impractical if a large number of proteins are to be tested for T cell reactivity. Since Th1 cells recognize mycobacterial antigens and epitopes in association with MHC class II molecules [46], an alternative approach has been to screen proteins for identification of regions that can associate with MHC molecules [61–64], and then test the peptides predicted to bind the MHC molecules for T cell reactivity. Such an approach drastically reduces the cost to screen for Th1 cell reactivity [65].

Although, several prediction programs have been proposed to identify peptides capable of binding to MHC molecules [66–68], virtual matrix-based prediction programs TEPITOPE and ProPred have been successfully employed to identify HLA-DR ligands derived from tumors and endogenous proteins involved in autoimmune diseases [69, 70]. In addition, these computer-assisted programs have previously been shown to accelerate research related to the design of vaccines and diagnostic tests through the identification of promiscuous peptides of mycobacterial proteins [71, 72]. These encouraging results have led researchers to investigate the use of the ProPred program to predict the HLA-DR binding sequences in 3 major antigenic proteins of *M. tuberculosis* encoded by RD1 region genes, i.e. PPE68, CFP10, and ESAT6, and to further confirm the prediction results in identification of promiscuous peptides by evaluating the predicted peptides for promiscuous presentation to T cells in humans [73].

The results suggested that some of the promiscuous peptides capable of Th1 cell reactivity in HLA heterogeneous populations can be predicted from the in silico HLA-DR binding analysis of complete proteins [73]. Therefore, a similar analysis was expanded to all 9 ORFs/proteins encoded by RD1 genes (table 2). The ProPred analysis of complete protein sequences of ORFs Rv3871 to Rv3879 was performed to identify HLA-promiscuous proteins for presentation to T cells. The results showed that all of the 9 ORFs of RD1 were promiscuous HLA-DR binders (table 3). The ProPred analysis further showed that several of them, i.e. Rv3871 and Rv3876 to Rv3879c, were predicted to bind all 51 (100%) HLA-DR alleles included in ProPred (table 3). The remaining ORFs, i.e. Rv3873 to Rv3875, were predicted to bind 84–98% of HLA-DR alleles included in ProPred (table 3). Furthermore, peptide pools of all of these ORFS were promiscuously recognized by peripheral blood mononuclear cells of appropriate donors (TB patients/BCG-vaccinated healthy subjects) (table 3). These results suggest that none of the RD1 protein sequences will have limitations in terms of binding to MHC class II molecules for presentation to Th1 cells. Hence, all of these proteins will not be limited due HLA-restriction, because the HLA-DR alleles included in ProPred are among those which are frequently expressed in different populations.

Identification of Major RD-Encoded Proteins Reactive in Cellular Assays

The protective immunity against *M. tuberculosis* is primarily mediated by cellular immunity (CMI) involving the interaction of antigen-specific Th1 cells and macro-

Table 3. HLA-DR binding prediction by ProPred analysis for the RD1 ORF protein sequence of Rv8371 to Rv3879 and their experimental validation in a Th1-cell assay

RD1 sequence	ProPred analysis for HLA-DR binding to										Positive in the Th1 assay, %
DRB	1.1	1.3	1.4	1.7	1.8	1.11	1.13	1.15	5.1	all alleles	
Alleles predicted to bind/alleles included in ProPred											
Rv3871	2/2	7/7	9/9	2/2	6/6	9/9	11/11	3/3	2/2	51/51 (100%)	53
Rv3872	2/2	6/7	9/9	0/2	3/6	9/9	10/11	2/3	2/2	43/51 (84%)	33
Rv3873	2/2	6/7	9/9	2/2	6/6	9/9	11/11	3/3	2/2	50/51 (98%)	68
Rv3874	2/2	7/7	9/9	0/2	5/6	9/9	10/11	0/3	2/2	44/51 (86%)	80
Rv3875	1/2	7/7	9/9	2/2	3/6	9/9	11/11	1/3	0/2	43/51 (84%)	48
Rv3976	2/2	7/7	9/9	2/2	6/6	9/9	11/11	3/3	2/2	51/51 (100%)	50
Rv3877	2/2	7/7	9/9	2/2	6/6	9/9	11/11	3/3	2/2	51/51 (100%)	33
Rv3978	2/2	7/7	9/9	2/2	5/6	9/9	11/11	2/3	2/2	49/51 (100%)	40
Rv3879c	2/2	7/7	9/9	2/2	6/6	9/9	11/11	3/3	2/2	51/51 (100%)	ND
MPT83P2[a]	1/2	0/7	0/9	0/2	1/6	0/9	1/11	0/3	0/2	3/51 (6%)	6
MPT83P14[b]	2/2	7/7	9/9	2/2	6/6	9/9	11/11	1/3	0/2	47/51 (92%)	65

ND = Not determined. [a] MPT83P2 nonpromiscuous peptide used as a negative control [60]. [b] MPT83P14 promiscuous peptide used as a positive control [60].

phages [74–78]. This interaction is often indicated by antigen-induced proliferation of T cells and is dependent on the interplay of cytokines secreted by these cells [79–81]. Although, a broad spectrum of cytokines may contribute to protection, the T helper type 1 (Th1) cytokines, dominated by interleukin (IL)-2 (responsible for proliferation of antigen-reactive T cells) and IFN-γ secretion, are considered principal mediators of protective immunity against TB [82–84]. Therefore, Th1 cell reactivity, indicated by antigen-induced cell proliferation and preferential secretion of IFN-γ has been used to identify antigens involved in protective immunity [85–90]. Hence, it was considered appropriate to test the proteins encoded by the RD genes of *M. tuberculosis* for these in vitro correlates of protective immunity to identify new candidates of RD-derived subunit vaccines against TB.

About a decade back, both recombinant proteins and overlapping synthetic peptides, corresponding to selected antigens of *M. tuberculosis*-specific RD1, were tested to identify antigens reactive in protective Th1 cell assays [91–94]. However, because of the ease and feasibility of getting the required peptides synthesized, recent studies have used 1,648 overlapping synthetic peptides covering all 86 ORFs predicted in 11 RDs deleted/absent in BCG (table 1). In these studies, CMI reactivities in response to peptide pools of each of the 11 RDs have been determined in relation to protective Th1-type responses by evaluating antigen-induced proliferation and secretion of IFN-γ using peripheral blood mononuclear cells obtained from pulmonary TB patients and healthy humans. The results showed that the highest Th1-responses were induced by peptide pools of RD1, RD7, and RD9 peptides [15, 16, 36, 37].

To identify individual proteins of Th1 cell-reactive RDs, further testing of the peptide pools corresponding to each protein of RD1, RD7, and RD9 has also been performed in Th1 cell assays. The results showed that 3 proteins of RD1, i.e. PPE68, ESAT6, and CFP10, and 2 proteins of RD7 (Rv2346c and Rv2947c) and RD9 (Rv3619 and Rv3620) were the best stimulators of Th1 cells in antigen-induced proliferation and/or IFN-γ secretion assays [41, 42, 95–97]. Among these proteins, ESAT6 and CFP10 were identified several years back as strong Th1-stimulating antigens using classical methods of protein purification from culture-filtrate of *M. tuberculosis,* and have been shown to have vaccine potential in animal models of TB [98–100]. However, ESAT6 and CFP10 are also antigens recommended for the specific diagnosis of active and latent TB [101–103] and are widely used for this purpose. Therefore, these antigens cannot be used as vaccines against TB. Hence, other Th1-stimulating antigens of RDs, i.e. PPE68, Rv2346c, Rv2947c, Rv3619, and Rv3620, must be investigated for protective efficacy in animals. If found promising in such studies, these antigens may be further explored in clinical trials as new-generation or alternative subunit vaccines against TB in humans.

Relevance of RD Proteins in Diagnosis Using Delayed-Type Hypersensitivity Responses in Guinea Pigs

The diagnostic potentials of 4 antigens encoded by *M. tuberculosis*-specific RD1 region genes (PE35, PPE68, CFP10, and ESAT-6) and RD9 region gene Rv3619c were further investigated for delayed-type hypersensitivity (DTH) skin responses in guinea pigs [104]. The recombinant *M. tuberculosis* proteins were expressed in *E. coli* and purified to homogeneity by affinity chromatography. Groups of guinea pigs were injected with heat-killed *M. tuberculosis* and live BCG, *M. avium,* and *M. vaccae*. Two to 4 weeks later, the guinea pigs were challenged intradermally in the flank region with mycobacterial sonicates and purified recombinant proteins. The DTH responses were quantitated by measuring erythema at the sites of injections after 24 h. The results showed that all mycobacterial sonicates induced positive DTH skin responses in *M. tuberculosis-, M. bovis* BCG-, *M. avium-,* and *M. vaccae*-injected guinea pigs. The purified proteins PE35, PPE68, CFP10, and ESAT-6 elicited positive DTH skin responses in the *M. tuberculosis*-injected group but not in BCG-, *M. avium-,* and *M. vaccae*-injected guinea pigs, whereas Rv3619c elicited positive DTH skin responses in *M. tuberculosis* and in BCG-injected groups but not in *M. avium-* and *M. vaccae*-injected guinea pigs. The overall results showed that the recombinant RD1 antigens induced *M. tuberculosis*-specific DTH skin responses [104–106]. Thus, theses antigens may be useful in the specific diagnosis of tuberculosis and therefore could provide an alternative to the currently used PPD-based DTH skin test.

Expression of RD Antigens in DNA Vaccine Vehicles and Immunological Evaluation of Recombinant Plasmids

RD1 *PE35, PPE68, EsxA, EsxB,* and RD9 *EsxV* genes were cloned into DNA vaccine vectors capable of expressing them in eukaryotic cells as fusion proteins, fused with immunostimulatory signal peptides of human IL-2 (hIL-2) and tissue plasminogen activator (tPA). The recombinant DNA vaccine constructs were evaluated for induction of antigen-specific cellular immune responses in mice. In brief, DNA corresponding to the above RD1 and RD9 genes were cloned into DNA vaccine plasmid vectors pUMVC6 and pUMVC7 (with hIL-2 and tPA signal peptides, respectively), and a total of 10 recombinant DNA vaccine constructs were obtained. BALB/c mice were immunized with the parent and recombinant plasmids and their spleen cells were tested for antigen-induced proliferation with antigens of *M. tuberculosis* and pure proteins corresponding to the cloned genes. The results showed that antigen-specific proliferation responses were observed for a given antigen only with spleen cells of mice immunized with the homologous recombinant DNA vaccine construct. The mice immunized with the parent plasmids did not show positive immune responses to any of the antigens of the cloned genes [107]. Mice immunized with the recombinant plasmid DNA (pUMVC6/PE35) showed positive Th1-type cellular responses to pure PE35, but not to an irrelevant antigen, i.e. PPE68 (Rv3873). However, the vaccine construct did not induce antigen-specific Th2-type (IL-5) or anti-inflammatory (IL-10) reactivity to PE35. Testing with synthetic peptides showed that Th1-type cells recognizing various epitopes of PE35 were induced in mice immunized with pUMVC6/PE35 DNA [108]. In another study, recombinant BCG expressing PE35 also induced Th1 responses in mice [109]. These results suggest that pUMVC6/PE35 may be useful as a safer vaccine candidate against TB.

Conclusion

Bioinformatics-based in silico methods for identification of expressed proteins and promiscuous antigens and peptides of *M. tuberculosis* RDs are useful to identify new candidates important for diagnosis and vaccine applications against TB. The experimental validation showed that several proteins and peptides encoded by genes in *M. tuberculosis*-specific RDs were immunodominant and could be useful for diagnostic applications using antibody and cell-mediated immunity-based assays. Two of these antigens (ESXA and ESXB) have been widely tested for diagnostic applications in vitro using IFN-γ assays and have shown diagnostic promise in countries with a low TB burden. *M. tuberculosis*-specific DTH skin responses are also observed with the selected antigens in animals immunized with *M. tuberculosis* and other mycobacteria. Furthermore, the usefulness of the identified antigens as new vaccine candidates was promising when they were expressed in DNA vaccine vectors. All of these results suggest that in silico analysis for immunodominant peptides and proteins could further be extended to include the complete proteome of *M. tuberculosis* to identify all possible candidates for diagnostic and vaccine applications against TB.

Acknowledgements

The work was supported by Kuwait University Research Sector grants MI02/12 and MI01/10 and by General Facility grant GM01/01.

Disclosure Statement

The author declares that no financial or other conflict of interest exists in relation to the content of the article.

References

1 World Health Organization: Global Tuberculosis Report 2012. http://www.who.int/tb/publications/global_report/.
2 Lönnroth K, Raviglione M: Global epidemiology of tuberculosis: prospects for control. Semin Respir Crit Care Med 2008;29:481–491.
3 Crampin AC, Glynn JR, Fine PE: What has Karonga taught us? Tuberculosis studied over three decades. Int J Tuberc Lung Dis 2009;13:153–164.
4 Moffitt MP, Wisinger DB: Tuberculosis: recommendations for screening, prevention, and treatment. Postgrad Med 1996;100:201–204.
5 Leung CC, Yew WW, Tam CM, et al: Tuberculin response in BCG vaccinated schoolchildren and the estimation of annual risk of infection in Hong Kong. Thorax 2005;60:124–129.
6 Mahadevan B, Mahadevan S, Serane VT, et al: Tuberculin reactivity in tuberculous meningitis. Indian J Pediatr 2005;72:213–215.
7 Andersen P, Doherty TM: The success and failure of BCG – implications for a novel tuberculosis vaccine. Nat Rev Microbiol 2005;3:656–662.
8 Castanon-Arreola M, Lopez-Vidal Y: A second-generation anti TB vaccine is long overdue. Ann Clin Microbiol Antimicrob 2004;3:10.
9 Hesseling AC, Caldwell J, Cotton MF: BCG vaccination in South African HIV-exposed infants – risks and benefits. S Afr Med J 2009;99:88–91.
10 Behr MA, Wilson MA, Gill WP, et al: Comparative genomics of BCG vaccines by whole-genome DNA microarray. Science 1999;284:1520–1523.
11 de Jonge MI, Brosch R, Brodin P, et al: Tuberculosis: from genome to vaccine. Expert Rev Vaccines 2005;4:541–551.
12 Amoudy HA, Al-Turab MB, Mustafa AS: Identification of transcriptionally active open reading frames within the RD1 genomic segment of *Mycobacterium tuberculosis*. Med Princ Pract 2006;15:137–144.
13 Hanif SNM, Al-Attiyah R, Mustafa AS: The natural expression of genes encoding major antigens of Rd1 and Rd9 in *M. tuberculosis* and other mycobacteria. Mycobact Dis 2011;1:105.
14 Mustafa AS: Biotechnology in the development of new vaccines and diagnostic reagents against tuberculosis. Curr Pharm Biotechnol 2001;2:157–173.
15 Mustafa AS: Development of new vaccines and diagnostic reagents against tuberculosis. Mol Immunol 2002;39:113–119.
16 Mustafa AS, Al-Attiyah R: *Mycobacterium tuberculosis* antigens and peptides as new vaccine candidates and immunodiagnostic reagents against tuberculosis. Kuwait Med J 2004;36:171–176.
17 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.
18 Ahmad S, Akbar PK, Wiker HG, et al: 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.
19 Ahmad S, Ali MM, Mustafa AS: Construction of a modified vector for efficient purification of recombinant *Mycobacterium tuberculosis* proteins expressed in *Escherichia coli*. Protein Expr Purif 2003;29:167–175.
20 Ahmad S, El-Shazly S, Mustafa AS, et al: 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.
21 Ahmad S, El-Shazly S, Mustafa AS, et al: The six mammalian cell entry proteins (Mce3A-F) encoded by the mce3 operon are expressed during in vitro growth of *Mycobacterium tuberculosis*. Scand J Immunol 2005;62:16–24.
22 Mustafa AS, Skeiky YA, Al-Attiyah R, et al: Immunogenicity of *Mycobacterium tuberculosis* antigens in *Mycobacterium bovis* BCG-vaccinated and *M. bovis*-infected cattle. Infect Immun 2006;74:4566–4572.
23 Amoudy HA, Ahmad S, Thole JE, et al: Demonstration of in vivo expression of a hypothetical open reading frame (ORF-14) encoded by the RD1 region of *Mycobacterium tuberculosis*. Scand J Immunol 2007;66:422–425.
24 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.
25 Hanif SNM, 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.
26 Mustafa AS: Recombinant and synthetic peptides to identify *Mycobacterium tuberculosis* antigens and epitopes of diagnostic and vaccine relevance. Tuberculosis (Edinb) 2005;85:367–376.
27 Oftung F, Geluk A, Lundin KEA, et al: Mapping of multiple HLA class II-restricted T-cell epitopes of the mycobacterial 70-kilodalton heat shock protein. Infect Immun 1994;62:5411–5418.
28 Ravn P, Demissie A, Eguale T, et al: Human T cell responses to the ESAT-6 antigen from *Mycobacterium tuberculosis*. J Infect Dis 1999;179:637–645.
29 Mustafa AS, Lundin KE, Meloen RH, et al: Identification of promiscuous epitopes from the mycobacterial 65-kilodalton heat shock protein recognized by human CD4(+) T cells of the *Mycobacterium leprae* memory repertoire. Infect Immun 1999;67:5683–5689.
30 Mustafa AS, Lundin KE, Meloen RH, et al: Cross-reactive epitopes and HLA-restriction elements in human T cell recognition of the *Mycobacterium leprae* 18-kD heat shock protein. Clin Exp Immunol 2000;120:85–92.
31 Mustafa AS, Cockle PJ, Shaban F, et al: Immunogenicity of *Mycobacterium tuberculosis* RD1 region gene products in infected cattle. Clin Exp Immunol 2002;130:37–42.
32 Al-Attiyah R, Mustafa AS, Abal AT, et al: Restoration of mycobacterial antigen-induced proliferation and interferon-gamma responses in peripheral blood mononuclear cells of tuberculosis patients upon effective chemotherapy. FEMS Immunol Med Microbiol 2003;38:249–256.
33 Mustafa AS, Oftung F, Amoudy HA, et al: Multiple epitopes from the *Mycobacterium tuberculosis* ESAT-6 antigen are recognized by antigen-specific human T cell lines. Clin Infect Dis 2000;30(suppl 3):S201–S205.
34 Al-Attiyah R, Shaban FA, Wiker HG, et al: Synthetic peptides identify promiscuous human Th1 cell epitopes of the secreted mycobacterial antigen MPB70. Infect Immun 2003;71:1953–1960.
35 Mustafa AS, Shaban FA, Al-Attiyah R, et al: Human Th1 cell lines recognize the *Mycobacterium tuberculosis* ESAT-6 antigen and its peptides in association with frequently expressed HLA class II molecules. Scand J Immunol 2003;57:125–134.

36 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.

37 Mustafa AS, Al-Attiyah R: Identification of *Mycobacterium tuberculosis*-specific genomic regions encoding antigens inducing protective cellular immune responses. Indian J Exp Biol 2009;47:498–504.

38 Al-Attiyah RJ, Mustafa AS: Mycobacterial antigen-induced cell proliferation and Th1 and Th2 cytokine secretion by peripheral blood mononuclear cells of diabetic and non-diabetic tuberculosis patients and *Mycobacterium bovis* BCG-vaccinated healthy subjects. Clin Exp Immunol 2009;158:64–73.

39 Mustafa AS, Al-Saidi F, El-Shamy ASM, et al: Cytokines in response to proteins predicted in genomic regions of difference of *Mycobacterium tuberculosis*. Microbiol Immunol 2011;55:267–278.

40 Al-Attiyah R, El-Shazly A, Mustafa AS: Comparative analysis of spontaneous and mycobacterial antigen-induced secretion of Th1, Th2 and pro-inflammatory cytokines by peripheral blood mononuclear cells of tuberculosis patients. Scand J Immunol 2012;75:623–632.

41 Mustafa AS: Vaccine potential of *Mycobacterium tuberculosis*-specific genomic regions: in vitro studies in humans. Expert Rev Vaccines 2009;8:1309–1312.

42 Mustafa AS: Cell mediated immunity assays identify proteins of diagnostic and vaccine potential from genomic regions of difference of *Mycobacterium tuberculosis*. Kuwait Med J 2010;42:98–105.

43 Al-Attiyah R, Mustafa AS: Characterization of human cellular immune responses to *Mycobacterium tuberculosis* proteins encoded by genes predicted in RD15 genomic region that is absent in *Mycobacterium bovis* BCG. FEMS Immunol Med Microbiol 2010;59:177–187.

44 Mustafa AS: What's new in the development of tuberculosis vaccines. Med Princ Pract 2012;21:195–196.

45 Mustafa AS: Proteins and peptides encoded by *M. tuberculosis*-specific genomic regions for immunological diagnosis of tuberculosis. Mycobac Dis 2012;2:e114.

46 Mustafa AS: HLA-restricted immune response to mycobacterial antigens: relevance to vaccine design. Hum Immunol 2000;61:166–171.

47 Al-Khodari NY, Al-Attiyah R, Mustafa AS: Identification, diagnostic potential and natural expression of immunodominant seroreactive peptides encoded by five *Mycobacterium tuberculosis*-specific genomic regions. Clin Vaccine Immunol 2011;18:477–482.

48 Zhang H, Wang J, Lei J, et al: PPE protein (Rv3425) from DNA segment RD11 of *Mycobacterium tuberculosis:* a potential B-cell antigen used for serological diagnosis to distinguish vaccinated controls from tuberculosis patients. Clin Microbiol Infect 2007;13:139–145.

49 Mukherjee P, Dutta M, Datta P, et al: The RD1-encoded antigen Rv3872 of *Mycobacterium tuberculosis* as a potential candidate for serodiagnosis of tuberculosis. Clin Microbiol Infect 2007;13:146–152.

50 Zhang MM, Zhao JW, Sun ZQ, et al: Identification of RD5-encoded *Mycobacterium tuberculosis* proteins as B-cell antigens used for serodiagnosis of tuberculosis. Clin Dev Immunol 2012;2012:738043.

51 Arend SM, van Meijgaarden KE, de Boer K, et al: Tuberculin skin testing and in vitro T cell responses to ESAT-6 and culture filtrate protein 10 after infection with *Mycobacterium marinum* or *M. kansasii*. J Infect Dis 2002;186:1797–1807.

52 Vordermeier HM, Whelan A, Cockle PJ, et al: Use of synthetic peptides derived from the antigens ESAT-6 and CFP-10 for differential diagnosis of bovine tuberculosis in cattle. Clin Diagn Lab Immunol 2001;8:571–578.

53 Olsen AW, Hansen PR, Holm A, et al: Efficient protection against *Mycobacterium tuberculosis* by vaccination with a single subdominant epitope from the ESAT-6 antigen. Eur J Immunol 2000;30:1724–1732.

54 Oftung F, Schinnick TM, Mustafa AS, et al: Heterogeneity among human T cell clones recognizing an HLA-DR4, Dw4 restricted epitope from the 18 kDa antigen of *Mycobacterium leprae* defined by synthetic peptides. J Immunol 1990;144:1478–1483.

55 Mustafa AS, Oftung F: Long lasting T cell reactivity to *M. leprae* antigens in human volunteers vaccinated with killed *M. leprae*. Vaccine 1993;11:1108–1112.

56 Mustafa AS, Shaban FA, Abal AT, et al: Identification and HLA restriction of naturally derived Th1-cell epitopes from the secreted *Mycobacterium tuberculosis* antigen 85B recognized by antigen-specific human CD4(+) T-cell lines. Infect Immun 2000;68:3933–3940.

57 Al-Attiyah R, Mustafa AS: Computer-assisted prediction of HLA-DR binding and experimental analysis for human promiscuous Th1 cell epitopes in a novel 24-kDa secreted lipoprotein (LppX) of *Mycobacterium tuberculosis*. Scand J Immunol 2004;59:16–24.

58 Mustafa AS, Shaban F: Mapping of Th1-cell epitope regions of *Mycobacterium tuberculosis* protein MPT64 (Rv1980c) using synthetic peptides and T-cell lines from *M. tuberculosis*-infected healthy humans. Med Princ Pract 2010;19:122–128.

59 Mustafa AS: In silico binding predictions for identification of HLA-DR-promiscuous regions and epitopes of *Mycobacterium tuberculosis* protein MPT64 (Rv1980c) and their recognition by human Th1 cells. Med Princ Pract 2010;19:367–372.

60 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.

61 De Groot AS, Jesdale B, Szu E, et al: An interactive web site providing major histocompatibility ligand predictions: application to HIV research. AIDS Res Hum Retroviruses 1997;13:529–531.

62 Hammer J, Bono E, Gallazzi F, et al: Precise prediction of major histocompatibility complex class-II peptide interaction based on peptide side chain scanning. J Exp Med 1994;180:2353–2358.

63 Sturniolo T, Bono E, Ding J, et al: Generation of tissue-specific and promiscuous HLA ligand data base using DNA microarrays and virtual HLA class II matrices. Nat Biotech 1999;17:555–561.

64 Singh H, Raghava GPS: ProPred: prediction of HLA-DR binding sites. Bioinformatics 2001;17:1236–1237.

65 Hagmann M: Computers aid vaccine design. Science 2000;290:80–82.

66 Zhang GL, Khan AM, Srinivasan KN, et al: MULTIPRED: a computational system for prediction of promiscuous HLA binding peptides. Nucleic Acids Res 2005;33:W172–W179.

67 Zhang L, Chen Y, Wong HS, et al: TEPITOPEpan: extending TEPITOPE for peptide binding prediction covering over 700 HLA-DR molecules. PLoS One 2012;7:e30483.

68 Oyarzún P, Ellis JJ, Bodén M, et al: PREDIVAC: CD4+ T-cell epitope prediction for vaccine design that covers 95% of HLA class II DR protein diversity. BMC Bioinformatics 2013;14:52.

69 Manici S, Sturniolo T, Imro MA, et al: Melanoma cells present a MAGE-3 epitope to CD4+ cytotoxic T cells in association with histocompatibility leucocyte antigen DR11. J Exp Med 1999;189:871–876.

70 Hammer J, Sturniolo T, Sinigaglia F: HLA class II peptide binding specificity and autoimmunity. Adv Immunol 1997;66:67–100.

71 Panigada M, Sturniolo T, Besozzi G, et al: Identification of a promiscuous T-cell epitope in *Mycobacterium tuberculosis* Mce proteins. Infect Immun 2002;70:79–85.

72 Vordermeier M, Whelan AO, Hewinson RG: Recognition of mycobacterial epitopes by T cells across mammalian species and use of a program that predicts human HLA-DR binding peptides to predict bovine epitopes. Infect Immun 2003;71:1980–1987.

73 Mustafa AS, Shaban FA: ProPred analysis and experimental evaluation of promiscuous Th1 cell epitopes of three major secreted antigens of *Mycobacterium tuberculosis*. Tuberculosis (Edinb) 2006;86:115–124.

74 Mustafa AS, Al-Attiyah R: Tuberculosis: looking beyond BCG vaccines. J Postgrad Med 2003;49:134–140.

75 Flynn J: Immunology of tuberculosis and implications in vaccine development. Tuberculosis 2004;4:93–101.
76 Boom WH, Canaday DH, Fulton SA, et al: Human immunity to *M. tuberculosis:* T cell subsets and antigen processing. Tuberculosis (Edinb) 2003;83:98–106.
77 Raja A: Immunology of tuberculosis. Indian J Med Res 2004;120:213–232.
78 Mahan CS, Zalwango S, Thiel BA, et al: Innate and adaptive immune responses during acute *M. tuberculosis* infection in adult household contacts in Kampala, Uganda. Am J Trop Med Hyg 2012;86:690–697.
79 Al-Attiyah R, Madi N, El-Shamy AS: Cytokine profiles in tuberculosis patients and healthy subjects in response to complex and single antigens of *Mycobacterium tuberculosis.* FEMS Immunol Med Microbiol 2006;47:254–261.
80 Liebeschuetz S, Bamber S, Ewer K, et al: Diagnosis of tuberculosis in South African children with a T-cell-based assay: a prospective cohort study. Lancet 2004;364:2196–2203.
81 Pinto LM, Grenier J, Schumacher SG, et al: Immunodiagnosis of tuberculosis: state of the art. Med Princ Pract 2012;2:4–13.
82 Bai X, Wilson SE, Chmura K, et al: Morphometric analysis of Th(1) and Th(2) cytokine expression in human pulmonary tuberculosis. Tuberculosis 2004;84:375–385.
83 Jo E-L, Park J-K, Dockrell HM: Dynamics of cytokine generation in patients with active pulmonary tuberculosis. Curr Opin Infect Dis 2003;16:205–210.
84 Derrick SC, Yabe IM, Yang A, et al: Vaccine-induced anti-tuberculosis protective immunity in mice correlates with the magnitude and quality of multifunctional CD4 T cells. Vaccine 2011;29:2902–2909.
85 Mustafa AS, Lundin KE, Meloen RH, et al: Identification of promiscuous epitopes from the mycobacterial 65-kilodalton heat shock protein recognized by human CD4(+) T cells of the *Mycobacterium leprae* memory repertoire. Infect Immun 1999;67:5683–5689.
86 Mustafa AS, Lundin KE, Oftung F: Isolation of recombinant phage clones expressing mycobacterial T cell antigens by screening a recombinant DNA library with human CD4+ Th1 clones. FEMS Immunol Med Microbiol 1998;22:205–216.
87 Al-Attiyah R, Mustafa AS, Abal AT, et al: In vitro cellular immune responses to complex and newly defined recombinant antigens of *Mycobacterium tuberculosis.* Clin Exp Immunol 2004;138:139–144.
88 Mustafa AS, Abal AT, Shaban F, et al: HLA-DR binding prediction and experimental evaluation of T-cell epitopes of mycolyl transferase 85B (Ag85B), a major secreted antigen of *Mycobacterium tuberculosis.* Med Princ Pract 2005;14:140–146.
89 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.
90 Mustafa AS: HLA-promiscuous Th1-cell reactivity of MPT64 (Rv1980c), a major secreted antigen of *Mycobacterium tuberculosis,* in healthy subjects. Med Princ Pract 2009;18:385–392.
91 Mustafa AS, Amoudy HA, Wiker HG, et al: Comparison of antigen-specific T-cell responses of tuberculosis patients using complex or single antigens of *Mycobacterium tuberculosis.* Scand J Immunol 1998;48:535–543.
92 Weldingh K, Andersen P: Immunological evaluation of novel *Mycobacterium tuberculosis* culture filtrate proteins. FEMS Immunol Med Microbiol 1999;23:159–164.
93 Mazurek GH, LoBue PA, Daley CL, et al: Comparison of a whole-blood interferon gamma assay with tuberculin skin testing for detecting latent *Mycobacterium tuberculosis* infection. JAMA 2001;286:1740–1747.
94 Katti MK: Assessment of RD 1-encoded mycobacterial antigens in the immunodiagnosis of pulmonary, extrapulmonary, and latent tuberculosis infections. J Infect Dis 2001;184:1497–1498.
95 Hanif SNM, El-Shammy AM, Al-Attiyah R, et al: Whole blood assays to identify Th1 cell antigens and peptides encoded by *Mycobacterium tuberculosis*-specific RD1 genes. Med Princ Pract 2008;17:244–249.
96 Mustafa AS, El-Shamy AM, Madi NM, et al: Cell-mediated immune responses to complex and single mycobacterial antigens in tuberculosis patients with diabetes. Med Princ Pract 2008;17:325–330.
97 Mustafa AS, Al-Attiyah R, Hanif SNM, et al: 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.
98 Zhang H, Shi CH, Xue Y, et al: Immune response and protective efficacy induced by fusion protein ESAT6-CFP10 of *M. tuberculosis* in mice. Xi Bao Yu Fen Zi Mian Yi Xue Za Zhi 2006;22:443–446.
99 Bai Y, Xue Y, Gao H, et al: Expression and purification of *Mycobacterium tuberculosis* ESAT-6 and MPT64 fusion protein and its immunoprophylactic potential in mouse model. Protein Expr Purif 2008;59:189–196.
100 Maue AC, Waters WR, Palmer MV, et al: An ESAT-6: CFP10 DNA vaccine administered in conjunction with *Mycobacterium bovis* BCG confers protection to cattle challenged with virulent *M. bovis.* Vaccine 2007;25:4735–4746.
101 Andersen P, Munk ME, Pollock JM, et al: Specific immune-based diagnosis of tuberculosis. Lancet 2000;356:1099–1104.
102 van Pinxteren LA, Ravn P, Agger EM, et al: Diagnosis of tuberculosis based on the two specific antigens ESAT-6 and CFP10. Clin Diagn Lab Immunol 2000;7:155–160.
103 Lalvani A, Nagvenkar P, Udwadia Z, et al: Enumeration of T cells specific for RD1-encoded antigens suggests a high prevalence of latent *Mycobacterium* tuberculosis infection in healthy urban Indians. J Infect Dis 2001;183:469–477.
104 Hanif SNM, Al-Attiyah R, Mustafa AS: Species-specific antigenic *Mycobacterium tuberculosis* proteins as tested by delayed-type hypersensitivity response. Int J Tuberc Lung Dis 2010;14:489–494.
105 Kalra M, Khuller GK, Sheikh JA, et al: Evaluation of *Mycobacterium tuberculosis* specific RD antigens for delayed type hypersensitivity responses in guinea pig. Indian J Exp Biol 2010;48:117–123.
106 Elhay MJ, Oettinger T, Andersen P: Delayed-type hypersensitivity responses to ESAT-6 and MPT64 from *Mycobacterium tuberculosis* in the guinea pig. Infect Immun 1998;66:3454–3456.
107 Hanif SNM, Al-Attiyah R, Mustafa AS: DNA vaccine constructs expressing *Mycobacterium tuberculosis*-specific genes induce immune responses. Scand J Immunol 2010;72:408–415.
108 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.
109 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;20:1230–1237.