The Brazilian Journal of
INFECTIOUS DISEASES

www.elsevier.com/locate/bjid

Brief Communication

Rational design of diagnostic and vaccination strategies for tuberculosis

Sibele Borsuk[a]*, *Fabiana Kommling Seixas*[a], *Daniela Fernandes Ramos*[a], *Tom Mendum*[b], *Johnjoe McFadden*[b], *Odir Dellagostin*[a]

[a]*Biotechnology Center, Universidade Federal de Pelotas, Pelotas, RS, Brazil*
[b]*School of Biomedical and Life Sciences, University of Surrey, Guildford, Surrey, UK*

ARTICLE INFO

Article history:
Received 26 April 2011
Accepted 16 July 2011

Keywords:
Gene knockout techniques
Clinical diagnosis
BCG vaccine

ABSTRACT

The development of diagnostic tests which can readily differentiate between vaccinated and tuberculosis-infected individuals is crucial for the wider utilization of bacillus Calmette-Guérin (BCG) as vaccine in humans and animals. BCG_0092 is an antigen that elicits specific delayed type hypersensitivity reactions similar in size and morphological aspects to that elicited by purified protein derivative, in both animals and humans infected with the tubercle bacilli. We carried out bioinformatics analyses of the BCG_0092 and designed a diagnostic test by using the predicted MHC class I epitopes. In addition, we performed a knockout of this gene by homologous recombination in the BCG vaccine strain to allow differentiation of vaccinated from infected individuals. For that, the flanking sequences of the target gene *(BCG_0092)* were cloned into a suicide vector. Spontaneous double crossovers, which result in wild type revertants or knockouts were selected using *SacB*. BCG_0092 is present only in members of the *Mycobacterium tuberculosis* complex. Eight predicted MHC class I epitopes with potential for immunological diagnosis were defined, allowing the design of a specific diagnostic test. The strategy used to delete the *(BCG_0092)* gene from BCG was successful. The knockout genotype was confirmed by PCR and by Southern blot. The mutant BCG strain has the potential of inducing protection against tuberculosis without interfering with the diagnostic test based on the use of selected epitopes from BCG_0092.

© 2012 Elsevier Editora Ltda. All rights reserved.

Introduction

Tuberculosis (TB) in humans and animals continues to cause major health problems on a global scale.[1] An important control strategy for the prevention of this disease is the use of effective vaccines. The *Mycobacterium bovis* bacillus Calmette-Guérin (BCG) vaccine has been widely used for controlling human tuberculosis.[2,3] However, a major constraint in the use of attenuated mycobacterial vaccines such as BCG is that vaccination interferes in the TB diagnosis.

The acid fast bacilli smear microscopy and culture on Löwenstein-Jensen medium are still the "gold standards" for the diagnosis of active TB.[4] However, the detection of extrapulmonary TB is often more difficult. An alternative

* *Corresponding author at:* Centro de Desenvolvimento Tecnológico, Universidade Federal de Pelotas, 354; 96010-900, 354, Pelotas, RS, 96010-900, Brazil
E-mail address: sibeleborsuk@gmail.com (Sibele Borsuk)
1413-8670/© 2012 Elsevier Editora Ltda. All rights reserved.

for rapid and cost-effective diagnostic methods for TB is molecular diagnosis using PCR[5,6] and immunodiagnosis, which uses the specific humoral and cellular immune responses of the host to infer the presence of infection or disease.[7,8] The tuberculin skin test (Mantoux) is a diagnostic test that has been used for more than 85 years.[9] However, the test lacks specificity for *M. tuberculosis/bovis* due to the following factors: 1) exposure to or infection with mycobacteria other than *M. tuberculosis/bovis*; 2) vaccination with BCG; and 3) presence in the purified protein derivative (PPD) of several antigens shared by *Mycobacterium* spp.[10] Tests based on PPD cannot distinguish between tuberculosis infection, *M. bovis* BCG vaccination or exposure to environmental mycobacteria.

More specific and sensitive alternatives to skin testing have been investigated. Detection of gamma-interferon (IFNγ) by sandwich ELISA has been tested.[11] Recombinant proteins MPB59, MPB64, MPB70 and ESAT 6 have all been tested in differential diagnostic tests based on IFNγ detection.[12,13]

A recombinant *M. tuberculosis* protein present in PPD that elicits strong and tuberculosis specific delayed type hypersensitivity (DTH) in guinea pigs has been identified and characterized.[14] The DPPD (corresponding to BCG_0092) protein is present only in tuberculosis complex mycobacteria and elicited DTH reactions in tuberculosis patients comparable to those elicited by tuberculin.[15] The results suggest that this molecule can be an important tool for a more specific diagnosis of tuberculosis in non-BCG vaccinated individuals;[15] however, it does not alleviate the problem with BCG vaccinated individuals.

Production of recombinant BCG_0092 proved to be difficult.[15] Synthetic peptides containing the major T cell epitopes of this protein could overcome the problem. Moreover, a BCG deficient in BCG_0092 would allow the use of this antigen as a tool for the diagnosis of tuberculosis infection even in BCG vaccinated individuals. BCG vaccination could then be carried out in humans and animals, without interfering with this new diagnostic test. In this work we performed bioinformatics analyses with the BCG_0092 sequence in order to identify major T cell epitopes and knocked out the *BCG_0092* gene from BCG by homologous recombination. The development of diagnostic tests which can differentiate vaccinated and tuberculosis-infected individuals could enhance the use of tuberculosis vaccines on a wider scale.

Material and methods

Sequence similarity search of BCG_0092 *gene*

To identify the presence of homologous genes, we did the BLASTP search and defined as homologous if (1) they had a FASTA E-score < e-10; (2) similarity was ≥ 50%; (3) the length of the alignable region between the two sequences was > 50% of the longer protein. This sequence was obtained directly from the National Center for Biotechnology Information (NCBI) using Nucleotide BLAST search. Settings were Mycobacterium Organism and PSI-BLAST Algorithm. Multiple alignments of each clusters were obtained by the program ClustalW2, followed by manual inspection and editing.[16]

Hydrophobicity

The BCG_0092 amino acid sequence was analyzed using the hydrophobicity indices determined by HPLC described by Cowan & Whittaker[17] using the Vector NTI Advance™[11] software to identify the hydrophobic regions presents in the protein.

MHC-I antigenic peptide prediction

The possible major histocompatibility complex I (MHC-I) immunogenic peptide epitopes for the antigen BCG_0092 and the proteasome cleavage prediction were computed by the program MHC-I Antigenic Peptide Processing Prediction (MAPPP), which is available on the website (http://www.mpiib-berlin.mpg.de/MAPPP/). A combination of cleavage algorithm to calculate the cleavages after a single residue was defined by FRAGPREDICT and PAProc with MHC binding matrices defined by SYFPEITHI and BIMAS in the expert mode of MAPPP were used.[18] Parameters for FRAGPREDICT were set at 0.6 (minimal residue cleavage and fragment cleavage probability) and at 0.3 in PAProc. We set all MHC-haplotypes and a range for length of peptide fragment of 8 to 10 amino acids. The threshold was set at an overall score of 0.9 expected for the gene product BCG_0092, for which we chose an overall score of 0.8. All peptides predicted were either 9- or- 10 mers.

Bacterial strains and media

M. bovis BCG Pasteur strain 1173P2 was used as a source of DNA and as the parent strain for deletion of the dppd gene (GenBank accession number NC_008769, locus_tag BCG_0092) by homologous recombination. The strain was cultured in Middlebrook 7H9 (Difco) liquid medium supplemented with 10% of oleic acid albumin/dextrose complex (OADC; Difco), 0.05% Tween 80, and Middlebrook 7H11 (Difco) solid medium supplemented with 10% OADC. Recombinants were selected on 7H11 medium supplemented with 20 μg/mL kanamycin, 100 μg/mL X-Gal and 10% of sucrose when necessary.

Knockout construction

Unmarked BCG Δ*BCG_0092* was constructed as previously described.[19] This strategy involves PCR amplifying chromosomal regions from either side of the gene of interest and cloning them into a suicide vector containing kanamycin resistance, *lacZ* and the counter selective *sacB* gene. When transformed into mycobacteria, the plasmid integrates into the chromosome by a single cross-over to give $LacZ^+$, Kan^R colonies. Spontaneous double crossovers, which result in wild type revertants or knock-outs, can then be selected by using *SacB*. The homologous chromosomal regions were PCR amplified from regions upstream (0091F- CGA GAA GCT TGA AGT TAC CAT CGG CAA GA and 0091R- ACT AGG ATC CCC TTA ACG ACG AGC CGG T) and downstream (0093F- AAT AGG ATC CGC CGC GGC AAT GAG T 0093R- CAT CTT AAT TAA TTG AGC CTG GCG GCC ATT) of the BCG Pasteur. These were cloned into p2-NIL using *Hind*III/*Bam*HI and *Bam*HI/*Pac*I restriction sites. The resulting plasmid was ligated to the pGOAL-19

PacI fragment[20] generating the plasmid named pGOAL-19/*BCG_0091.0093*. To transform the knockout plasmids into mycobacteria, cultures in logarithmic growth at OD_{600} of 0.6-1 were harvested by centrifugation. The pellet was washed in ´1, ´0.5 and ´0.2 volumes of 10% glycerol (37°C for BCG) and the cells were then resuspended in ´0.01 volume. A 200 mL aliquot of competent cells were transformed by electroporation (25 mF, 1000 W, 2.5 V) with approximately 1 µg UV irradiated (100 mJ/cm^2) plasmid. BCG cells were recovered in 10 mL 7H9 for 12-24 h and plated on 7H11 with kanamycin and X-Gal. Colonies with β-galactosidase activity were sub-cultured onto plates containing X-Gal and 10% sucrose. White colonies were selected and their genotype determined by PCR using 0091-F and 0093-R, and by Southern blot using probes containing the homologous regions, the *BCG_0092* gene and pGOAL-19.

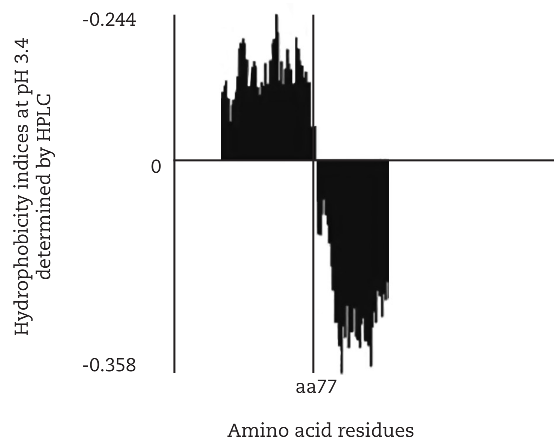

Fig. 1 - Hydrophobicity of the amino acid sequence of BCG_0092. Above baseline are the highly hydrophobic regions and below baseline the regions with low hydrophobicity.

Results

Sequence similarity search of BCG_0092 *gene*

The similarities between the *M. tuberculosis BCG_0092* gene and their orthologues in other mycobacterial species, especially environmental mycobacteria, were analyzed using the ClustalW2.[16] Clustal W2 analysis revealed the presence of *BCG_0092* in four mycobacterial species (*M. tuberculosis, M. bovis, M. marinum, M. ulcerans*). The analysis showed that the identity of *BCG_0092* gene among *M. tuberculosis* complex species (*M. tuberculosis, M. bovis* and *M. bovis* BCG) is 100%. Orthologues of the gene are also present in *M. marinum* and *M. ulcerans*, two slow-growing cutaneous pathogens; however, the nucleotide sequence has an identity of only 79%. This shows that the use of BCG_0092 protein in a diagnostic test can result in a specific diagnosis of tuberculosis.

Hydrophobicity

The BCG_0092 amino acid sequence was analyzed to identify the hydrophobic regions present in the protein using the hydrophobicity indices determined by HPLC described by Cowan & Whittaker[17] using the Vector NTI Advance™ 11software (Invitrogen). The first 77 amino acid contain highly hydrophobic residues (Fig. 1).

MHC-I antigenic peptide prediction

The cleavage probability of specific fragments from BCG_0092 protein was determined by MAPPP.[21] For that the probability of a cut after each residue within the sequence and the cleavage probability for all possible fragments between two cut-sites and with the right length were calculated. The cleavage probability for a fragment depends on the probability of both the N- and the C-residue, as well as the probabilities of the residues between these sites. The flanking regions to the left and the right of a fragment are considered. Twenty-nine fragments from BCG_0092 were identified (data not shown). However, 17 residues had the score above 0.9.

The prediction also was performed by MAPPP[21] which combines existing prediction tools for proteasomal processing and MHC class I anchoring. The prediction of the peptides binding to MHC class I molecules is based on a score calculated for each subsequence. Each amino acid at a specific position within a subsequence is given a value. Depending on the algorithms selected in the form, the values for the 8 to 10 amino acids are then multiplied (BIMAS) or added (SYFPEITHI) to determine the score for the subsequence. These values have been pre-calculated and stored in static matrices. The pre-calculation was done by algorithms and background data tables at BIMAS and SYFPEITHI[22] matrices.

We considered the epitopes when the MHC bound epitope evolved from a proteasomal cleaved fragment with exactly the same size and when it was trimmed at the n-terminal side to get the epitope (with a mechanism already known to exist). Eight predicted antigenic peptides from BCG_0092 have the highest probability to be processed and finally presented on the cell surfaces. The alleles that have the possibility of binding to the peptides from BCG_0092 belonged to HLA-A (human leukocyte antigen) and HLA-B.

Knockout construction

The sequences upstream and downstream of the target gene were cloned into a suicide vector. The plasmid containing the sequences that flank the *BCG_0092* gene was named pGOAL-19/BCG_0091.0093. Unmarked *M. bovis* BCG *ΔBCG_0092* was successfully constructed by homologous recombination using the strategy described by Parish and Stoker.[20] The knockout genotype was confirmed by PCR using primers that anneal to a sequence flanking the knockout target. The knockout genotype allowed amplification of a fragment of 1,822 bp, while the

wild BCG resulted in the amplification of a 2,248 bp fragment (Fig. 2). PCR using the BCG_0092 gene as target resulted in no amplification, further confirming the knockout genotype (data not shown). Southern blot probing with *BCG_0092* gene and pGOAL-19 also confirmed the knockout genotype (data not shown).

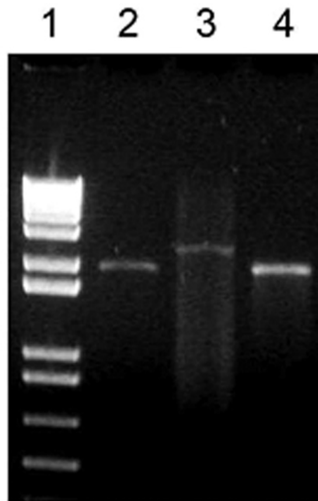

Fig. 2 - Agarose gel electrophoresis of PCR products using primers flanking the BCG_0092 gene. Lane 1, 1 Kb DNA ladder (Invitrogen); lane 2, the BCG_0092 knockout mutant -BCG PasteurΔBCG_0092; lane 3, BCG WT (negative control); lane 4, pGOAL-19/BCG_0091.0093 (positive control).

Discussion

To perform a rational design of a new TB diagnostic strategy, bioinformatics analyses of BCG_0092 protein were carried out. We evaluated (1) the presence and relatedness of this protein among *Mycobacterium* spp.; (2) the hydrophobicity properties of the protein; and (3) the presence of predicted dominant MHC class I epitopes.

The similarity analysis has been possible because there are presently 14 complete mycobacterial genome sequences deposited in GenBank, representing nine species: *M. tuberculosis; M. bovis; M. avium; M. smegmatis; M. ulcerans; M. gilvum; M. leprae; M. vanbaalenii; M. marinum; M. abscessus*.[23-28] Blast analysis with the BCG_0092 protein sequence revealed no match with *M. tuberculosis* H37Rv. When a careful analysis was carried out, an error in the annotation of the genome of several members of the *M. tuberculosis* complex was identified. The annotated coding sequence for this gene uses an alternative start codon, resulting in a distinct amino acid sequence for the Rv0061 (BCG_0092), the coding sequence for the DPPD protein.

In 2000, a study performed Southern blot analysis with DNA from a few species of mycobacteria and the DPPD gene (*BCG_0092*) was shown to be present in *M. tuberculosis* and in *M. bovis* BCG.[14] At that time, only a few mycobacterial genomes had been sequenced. The in silico analysis that we carried out confirms the results obtained by Coler et al.[14] and adds information regarding the analysis of 14 other genome sequences.

The identification of hydrophobic domains and prediction of binding of MHC I molecules to peptides is important to the development of rational design diagnosis. The hydrophobic interactions are the most important non-covalent forces that are responsible for different phenomena such as structure stabilization of proteins,[18] binding of enzymes to substrates,[29] and folding of proteins.[30] In a previous work, Coler et al.[14] tested several alternatives for expression of BCG_0092 (named DPPD by the authors). However, none of these systems provided reasonable expression of this protein. To overcome this problem, the DPPD was expressed as a chimeric construct with a 14-kDa C-terminal fragment of the *M. tuberculosis* protein named Ra12.[14]

The prediction of binding of MHC I molecule to a peptide shows the ability of binding of MHC molecules, which will transport the peptide to the cell surface.[31] In an infection with *M. tuberculosis* the protein BCG_0092, have cleavage probability in specific fragment, some of these residues have the possibility of binding to MHC I molecules and as a consequence the generation of BCG_0092 specific immune response.

Based on the hydrophobicity analysis and the identification of predicted MHC epitopes, we suggest the cloning and expression of the C-terminal region of the BCG_0092 protein (from 77 amino acid to 141 amino acid). In this hydrophilic region five epitopes with ability of binding to MHC molecules were identified. The DPPD (corresponding to BCG_0092) protein was the only antigen that elicited DTH reactions in tuberculosis patients comparable to those elicited by tuberculin.[15] The results suggest that this molecule has the potential for the developing of more specific diagnostic tests for tuberculosis in non-BCG vaccinated individuals. An alternative is to use these five epitopes present in this region or the three present in the hydrophobic region, synthetically produced, in a cocktail of epitopes.[32]

Synthetic peptides representing fragments of ESAT-6, MPB64, MPB83, MPB70, and CFP-10 alone or in different combinations, have been described recently as useful in the detection of TB in cattle or humans,[12,19,33,34] in some cases with limited success.

A diagnostic test using peptides derived from BCG_0092 individually or as a cocktail, could improve the specificity and sensitivity of such test. This test could be based on the DTH reaction similar to the tuberculin test, or a test based on the detection of IFNγ production when PBMC (peripheral blood mononuclear cell) are sensitized with the BCG_0092-derived MHC epitopes.

The *BCG_0092* gene is expressed in *M. bovis* BCG. A diagnostic test using peptides derived from this antigen would react with vaccinated individuals, decreasing the specificity of the test. To allow the vaccination with BCG without interfering in the diagnostic test proposed in this work, we used a strategy for knocking out the *BCG_0092* open reading frame (Fig. 3).

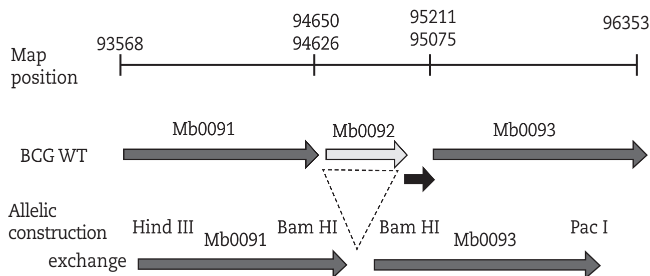

Fig. 3 - Alignment of the *M. bovis* genome at the locus (BCG_0092) with the DNA construct used for BCG_0092 knockout by homologous recombination.

The encouraging results obtained with use of DPPD (BCG_0092) in the skin test, with similar results to PPD in terms of sensitivity and with improved specificity[15] prompted us to investigate the possibility of genetically modifying the BCG strain in order to knock out this antigen. This BCG *Δbcg_0092* strain will be evaluated in animal models of tuberculosis to ascertain that the protective properties of the BCG vaccine have not been compromised. It is expected that this mutant strain will be able to protect against the severe forms of tuberculosis without sensitizing the animal against the BCG_0092 antigen. If this hypothesis is confirmed, a new perspective for vaccination of farm animals with BCG will be established. This will provide not only protection against tuberculosis but also the possibility of using BCG as a multivalent vaccine vector.[2]

The results demonstrated that dominant MHC epitopes of carefully selected mycobacteria complex-specific protein can be used to devise a simple peptide-based immunodiagnostic test. Moreover, the knockout BCG strain could allow differentiation between vaccinated and infected individuals. These findings support the systematic application of bioinformatics tools in combination with in vitro methods for the rational design of diagnostic and vaccination strategies for TB.

Conflict of interest

All authors declare to have no conflict of interest.

REFERENCES

1. WHO. Global Tuberculosis Control. WHO report. WHO/CDS/TB/2002. 2002.
2. Bastos RG, Borsuk S, Seixas FK, Dellagostin OA. Recombinant *Mycobacterium bovis* BCG. Vaccine. 2009,;27:6495-503.
3. Bloom BR, Fine PEM. The BCG experience: implications for future vaccines against tuberculosis. in: B.R> Bloom (ed), Tuberculosis: pathogenicity, protection and control. American Society for Mycrobiology. 1994;531-57.
4. Jensen KA. Culture and type differentiation among strains of tubercle bacilli: a simplification of the methodology for application in laboratory practice. Int J Tuberc Lung Dis. 2008;12:1382-92.
5. Dziadek J, Sajduda A, Borun TM. Specificity of insertion sequence-based PCR assays for *Mycobacterium tuberculosis* complex. Int J Tuberc Lung Dis. 2001;5:569-74.
6. Parra A, Garcia N, Garcia A, et al. Development of a molecular diagnostic test applied to experimental abattoir surveillance on bovine tuberculosis. Vet Microbiol. 2008;127:315-24.
7. Gennaro ML. Immunologic diagnosis of tuberculosis. Clin Infect Dis. 2000;30:S243-6.
8. Minion J, Zwerling A, Pai M. Diagnostics for tuberculosis: what new knowledge did we gain through The International Journal of Tuberculosis and Lung Disease in 2008? Int J Tuberc Lung Dis. 2009;13:691-7.
9. Huebner RE, Schein MF, Bass JB, Jr. The tuberculin skin test. Clin Infect Dis. 1993;17:968-75.
10. Young DB. Heat-shock proteins: immunity and autoimmunity. Curr Opin Immunol. 1992;4:396-400.
11. Dalley D, Dave D, Lesellier S, et al. Development and evaluation of a gamma-interferon assay for tuberculosis in badgers (*Meles meles*). Tuberculosis. 2008;88:235-43.
12. Buddle BM, Parlane NA, Keen DL, et al. Differentiation between *Mycobacterium bovis* BCG-vaccinated and *M. bovis*-infected cattle by using recombinant mycobacterial antigens. Clin Diagn Lab Immunol. 1999;6:1-5.
13. Buddle BM, Ryan TJ, Pollock JM, et al. Use of ESAT-6 in the interferon-gamma test for diagnosis of bovine tuberculosis following skin testing. Vet Microbiol. 2001;80:37-46.
14. Coler RN, Skeiky YA, Ovendale PJ, et al. Cloning of a *Mycobacterium tuberculosis* gene encoding a purifed protein derivative protein that elicits strong tuberculosis-specific delayed-type hypersensitivity. J Infect Dis. 2000;182:224-33.
15. Campos-Neto A, Rodrigues-Junior V, Pedral-Sampaio DB et al. Evaluation of DPPD, a single recombinant *Mycobacterium tuberculosis* protein as an alternative antigen for the Mantoux test. Tuberculosis. 2001;81:353-8.
16. Larkin MA, Blackshields G, Brown NP et al. Clustal W and Clustal X version 2.0. Bioinformatics. 2007;23:2947-8.
17. Cowan R, Whittaker RG. Hydrophobicity indices for amino acid residues as determined by high-performance liquid chromatography. Pept Res. 1990;3:75-80.
18. Privalov PL, Gill SJ. Stability of protein structure and hydrophobic interaction. Adv Protein Chem. 1988, 39:191-234.
19. 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;8:571-8.
20. Parish T, Stoker NG. Use of a flexible cassette method togenerate a double unmarked *Mycobacterium tuberculosis* tlyA plcABC mutant by gene replacement. Microbiology. 2000;146:1969-75.

21. Hakenberg J, Nussbaum AK, Schild H, et al. MAPPP: MHC class I antigenic peptide processing prediction. Appl Bioinformatics. 2003;2:155-8.
22. Rammensee H, Bachmann J, Emmerich NP, et al. SYFPEITHI: database for MHC ligands and peptide motifs. Immunogenetics. 1999;50:213-9.
23. Cole ST, Brosch R, Parkhill J, et al. Deciphering the biology of *Mycobacterium tuberculosis* from the complete genome sequence. Nature. 1998;393:537-44.
24. Fleischmann RD, Alland D, Eisen JA, et al. Whole-genome comparison of *Mycobacterium tuberculosis* clinical and laboratory strains. J Bacteriol. 2002;184:5479-90.
25. Garnier T, Eiglmeier K, Camus JC, et al. The complete genome sequence of *Mycobacterium bovis*. Proc Natl Acad Sci USA. 2003;100:7877-82.
26. Seki M, Honda I, Fujita I, et al. Whole genome sequence analysis of *Mycobacterium bovis* bacillus Calmette-Guérin (BCG) Tokyo 172: a comparative study of BCG vaccine substrains. Vaccine. 2009;27:1710-6.
27. Stinear TP, Seemann T, Pidot S, et al. Reductive evolution and niche adaptation inferred from the genome of *Mycobacterium ulcerans*, the causative agent of Buruli ulcer. Genome Res. 2007;17:192-200.
28. Stinear TP, Seemann T, Harrison PF, et al. Insights from the complete genome sequence of *Mycobacterium marinum* on the evolution of *Mycobacterium tuberculosis*. Genome Res. 2008;18:729-41.
29. Erikkson LS, Conn HO. Branched-chain amino acids in hepatic encephalopathy. Gastroenterology. 1990;99:604-7.
30. Dill KA. Dominant forces in protein folding. Biochemistry. 1990;29:7133-55.
31. Parker KC, Bednarek MA, Coligan JE. Scheme for ranking potential HLA-A2 binding peptides based on independent binding of individual peptide side-chains. J Immunol. 1994;152:163-75.
32. Mollenkopf HJ, Grode L, Mattow J, et al. Application of mycobacterial proteomics to vaccine design: improved protection by *Mycobacterium bovis* BCG prime-Rv3407 DNA boost vaccination against tuberculosis. Infect Immun. 2004;72:6471-9.
33. 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;7:155-60.
34. Vordermeier HM, Cockle PC, Whelan A, et al. Development of diagnostic reagents to differentiate between *Mycobacterium bovis* BCG vaccination and *M. bovis* infection in cattle. Clin Diagn Lab Immunol. 1999;6:675-82.