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(54) **USE OF AMINO ACID SEQUENCES FROM MYCOBACTERIUM TUBERCULOSIS OR CORRESPONDING NUCLEIC ACIDS FOR DIAGNOSIS AND PREVENTION OF TUBERCULAR INFECTION, DIAGNOSTIC KIT AND VACCINE THEREFROM**

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USPC 435/7.1, 7.92, 6.12, 7.24, 7.32, 7.2, 34, 435/4, 287.2, 6.11, 6.15, 6.16, 287.1, 29, 5, 435/6.1, 7.21, 7.23, 7.94, 975
See application file for complete search history.

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(57) **ABSTRACT**

The present invention refers to the use of gene sequences or portions thereof characterized in that the same belong to the classes of in vitro and ex vivo induced, repressed or conserved genes in *Mycobacterium tuberculosis* currently infected human macrophages and to corresponding peptides or consensus peptides or proteins for the preparation of specific bio-markers for the diagnosis and prevention of active or latent disease.

4 Claims, 8 Drawing Sheets

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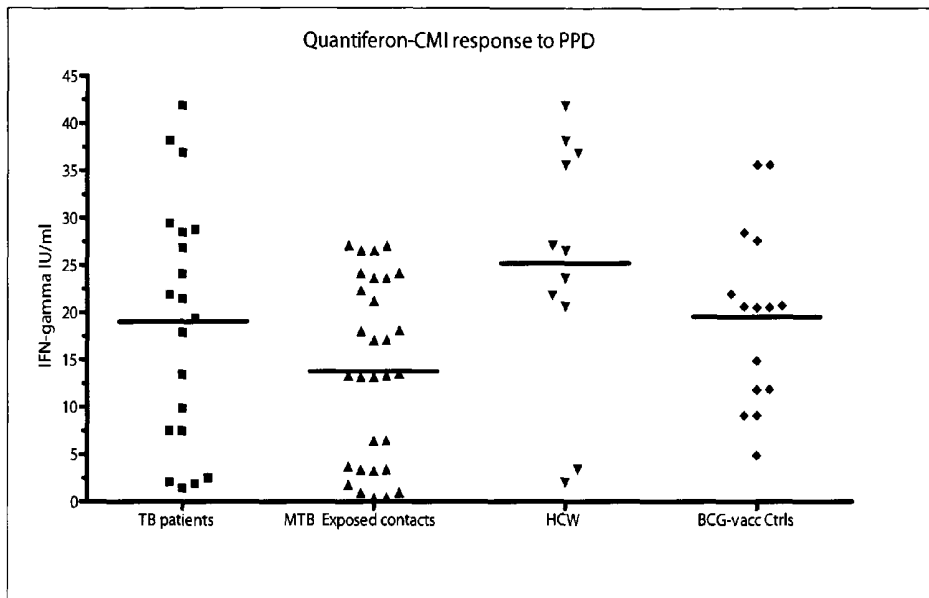


Fig.1

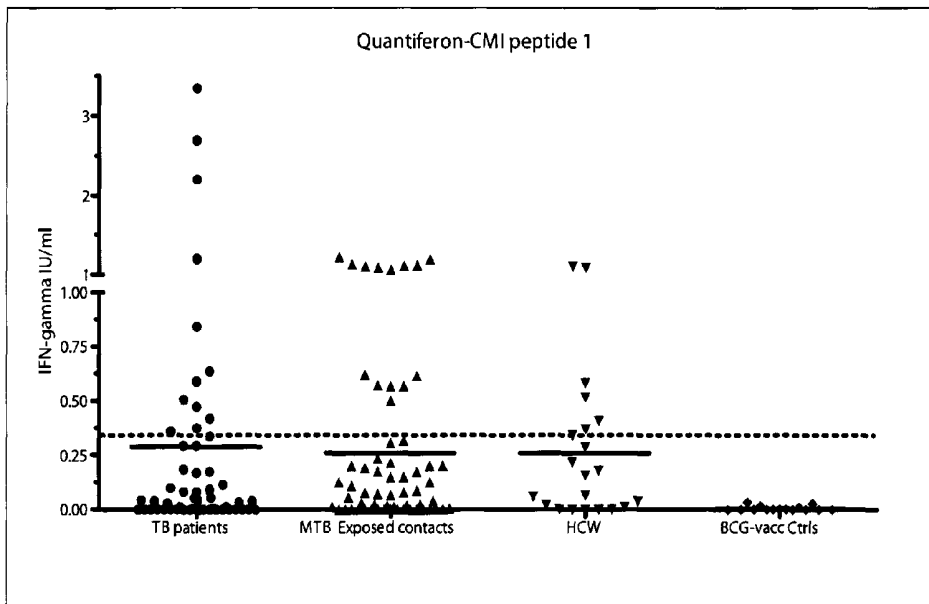


Fig.2

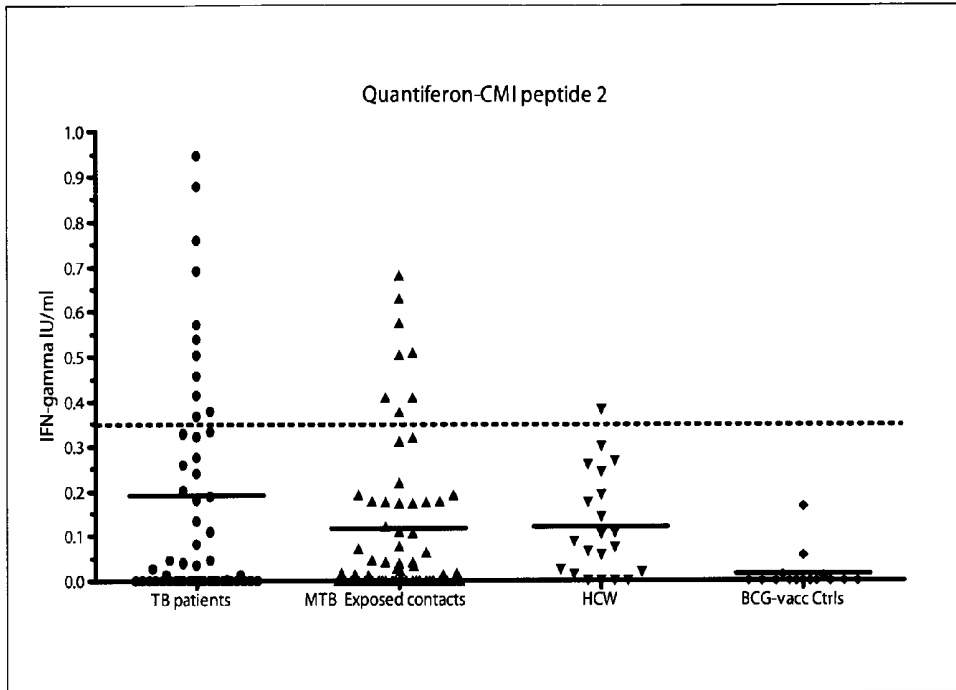


Fig.3

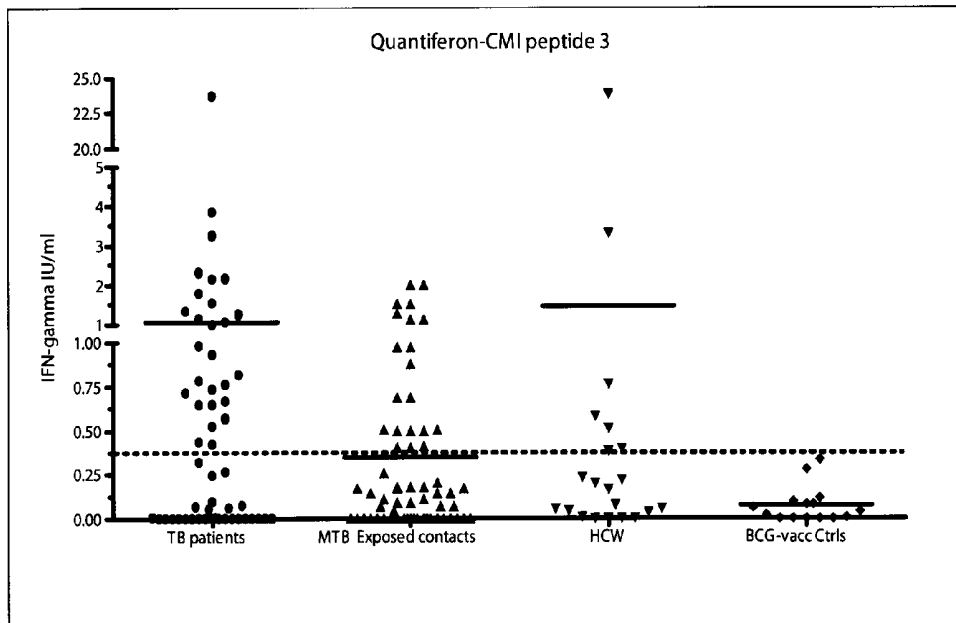


Fig.4

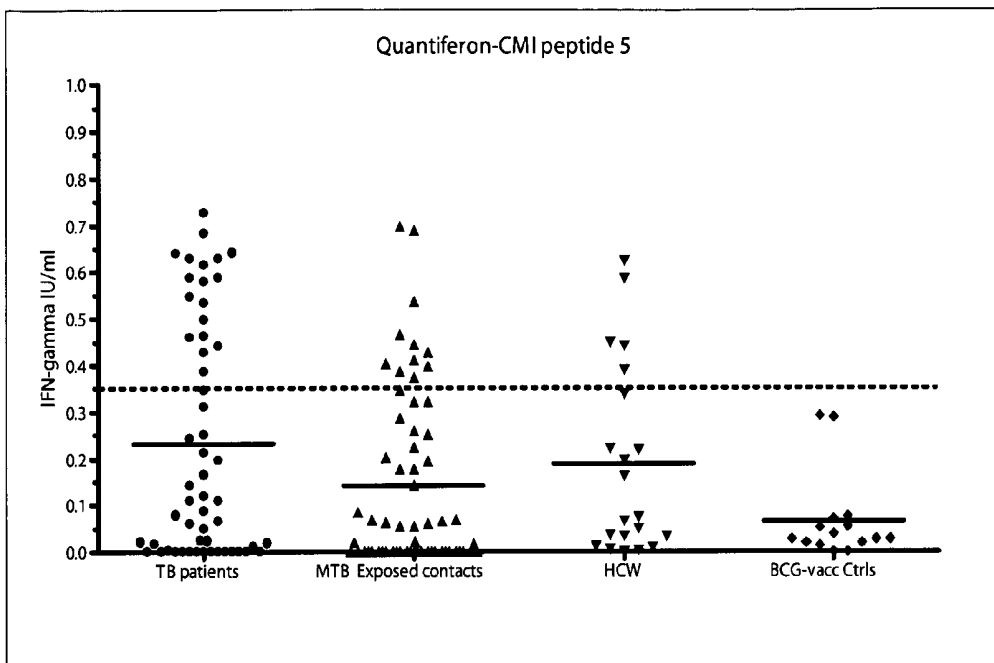


Fig.6

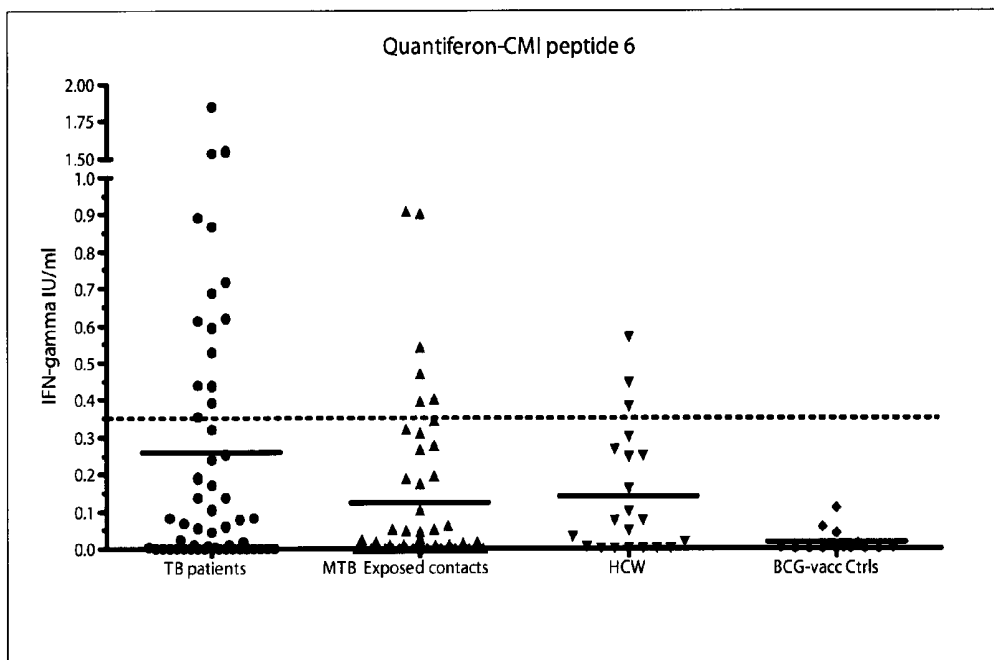


Fig.7

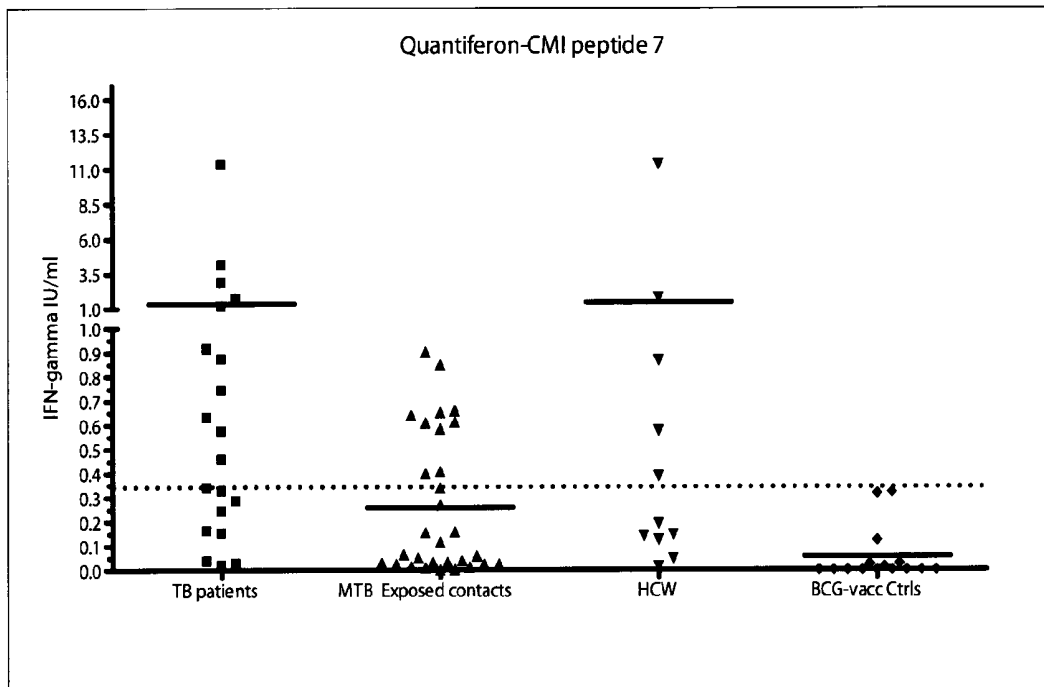


Fig.8

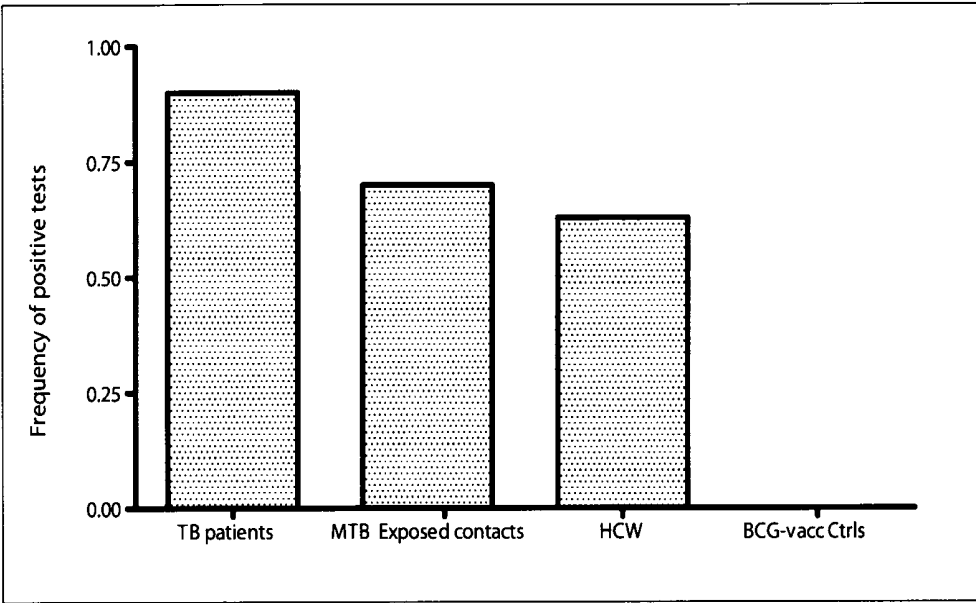


Fig.9

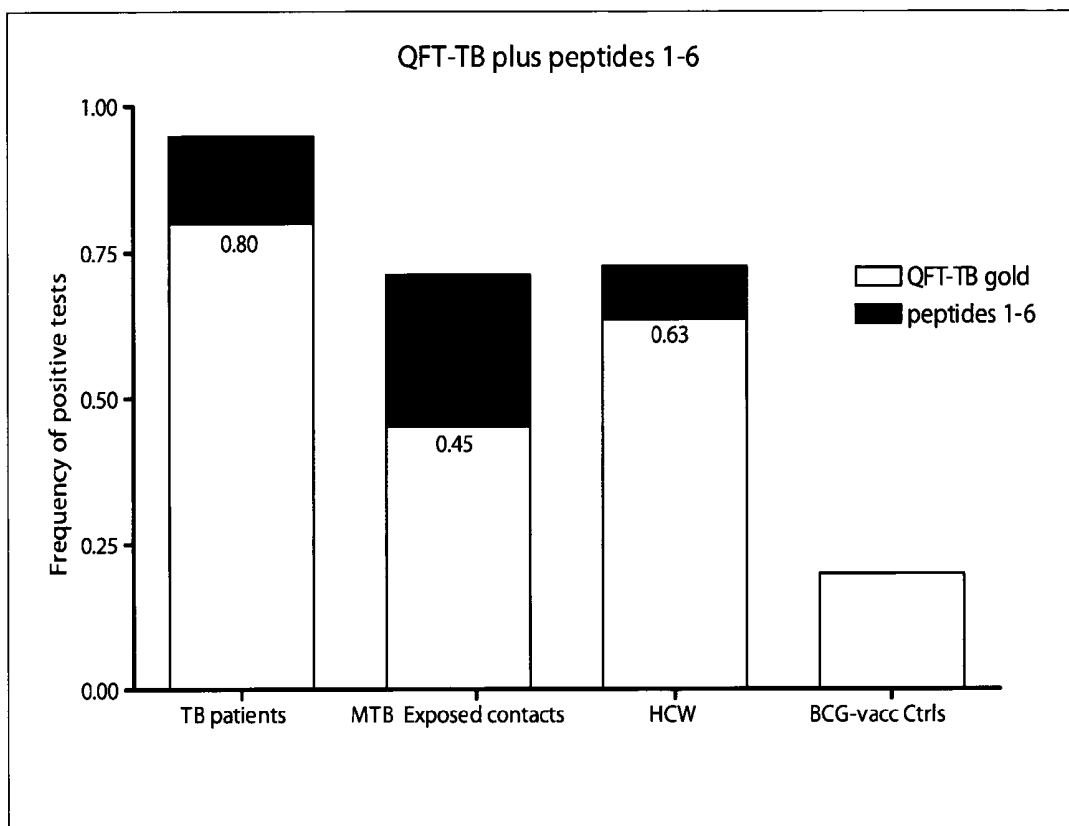


Fig.10

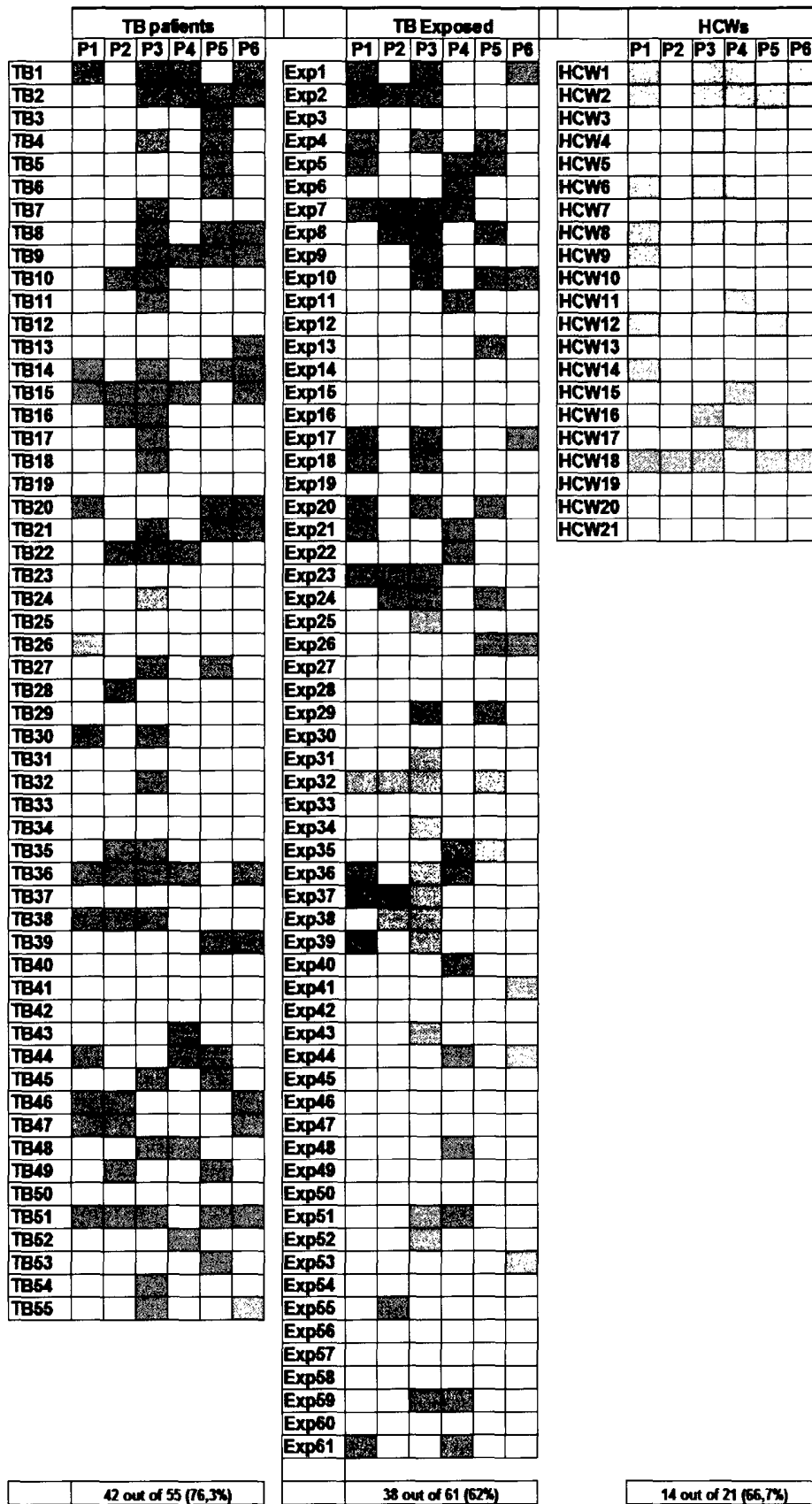


Fig.11

**USE OF AMINO ACID SEQUENCES FROM
MYCOBACTERIUM TUBERCULOSIS OR
CORRESPONDING NUCLEIC ACIDS FOR
DIAGNOSIS AND PREVENTION OF
TUBERCULAR INFECTION, DIAGNOSTIC
KIT AND VACCINE THEREFROM**

STATEMENT REGARDING SEQUENCE
LISTING

The Sequence Listing associated with this application is provided in text format in lieu of a paper copy, and is hereby incorporated by reference into the specification. The name of the text file containing the Sequence Listing is 200185_404USPC_SEQUENCE_LISTING.txt. The text file is 21 KB, was created on Mar. 28, 2013, and is being submitted electronically via EFS-Web.

The present invention concerns the use of amino acid sequences from *Mycobacterium tuberculosis* or corresponding nucleic acids thereof for diagnosis and prevention of tubercular infection, diagnostic kit and vaccine therefrom.

More particularly the invention refers to the use of gene sequences or portions thereof characterized in that the same belong to the classes of in vitro and ex vivo induced, repressed or conserved genes in *Mycobacterium tuberculosis* currently infected human macrophages and corresponding peptides or consensus peptides or proteins for the preparation of specific bio-markers for diagnosis and prevention of active or latent disease.

The laboratory diagnosis of tubercular infection and active disease onset is a very important in order to guarantee specificity, rapidity and effectiveness of the therapeutic treatment.

As to the active tubercular disease, current diagnostic protocols are based on microscopic, culture or molecular methods. With reference to microscopic examination, high mycobacteria concentrations in biological sample (from 5 to 10000/ml) are needed in order a positive result to be obtained and the sensitivity is generally lower than 60% with reduced specificity due to the inability of the test to discriminate MTB and not tubercular mycobacteria.

As to culture tests, the use thereof does not assure the medical report to be carried out within acceptable time period. In fact, MTB colonies are visible only 10-24 days after the seed thereof in solid or liquid culture media (1), as the bacterium grows slowly in vitro. Furthermore culture tests, although considered highly sensitive, produce about 10-30% rate of negative false results and are expensive.

Molecular biology tests are highly sensitive and specific, with a sensitivity comparable to culture tests and the ability of quickly discrimination of MTB and not tubercular mycobacteria. However, the sensitivity thereof is meaningfully lower for samples containing low mycobacteria concentration, that is those negatives to microscopic examination, moreover only highly specialized laboratories can use the same being thus very expensive.

Tuberculosis is still a world-wide public health emergency lacking necessary economic resources in order an efficient diagnosis and treatment program to be planned in high incidence countries and being hardly diagnosable in affected population segments in low incidence industrialized countries.

Currently, in high incidence countries, TB active diagnosis is based on MTB microscopic (40-90% sensitivity) and culture (70-90% sensitivity) examination with 2-6 week waiting times for culture tests. In low incidence industrialized countries, TB active diagnosis is based on microscopic and culture

tests and, for not tubercular MTB and mycobacteria discrimination or microscopically negative samples, on molecular tests (70-90% sensitivity).

As to the diagnosis of latent or not active tubercular infection, traditional diagnostic test is tuberculin skin test, an in vivo economic and quick diagnostic test that, standardized in years '50 allows the possibility the infection to be detected carefully and quickly thus allowing essential epidemiological surveys about tubercular infection incidence and prevalence to be carried out. Tuberculin test from the point of view of public health allowed infection incidence and prevalence to be monitored in order a global disease control to be obtained and, from the point of view of preventive and clinical medicine, infected contacts by active TB bearers to be identified allowing to establish therapeutic protections against tubercular infection aiming to prevent new case onsets. Therefore latent infection diagnosis is a fundamental element of the fight against the tuberculosis both in high and low incidence countries

Various cellular and molecular immunological issues demonstrated that the contact with MTB or antigens thereof in vitro elicits a strong cell-mediated response characterized by high production of interferon- γ (IFN- γ). This suggested that the identification of IFN- γ releasing T-lymphocytes or measure of cytokine itself, as response to the mycobacterium or antigen thereof, represents a way in order already occurred infection to be diagnosed equivalently to tuberculin test.

With respect to this issue recently two kits for tubercular infection diagnosis, i.e. QuantiFERON-TB Gold and T-SPOT TB, have been commercialized, said kits using proteins or peptides based on MTB genes, belonging to MTB genome RD1 differentiation region, in order to stimulate the IFN- γ production in T lymphocytes of circulating blood. The cost of kits is quite high in order to be used on wide scale in tuberculosis high incidence countries. The sensitivity of two commercial kits is similar, being sensitivity and specificity range from 70 to 90% and from 80 to 95%, respectively. (2). Moreover, sensitivity and specificity of these kits used in Tuberculosis high incidence areas as compared to traditionally used tuberculin test are still debated. In fact there are cases wherein the skin test proves to be even more sensitive than Quantiferon (3) and cases wherein the two tests are exactly comparable (4).

Major limits of still more widely used tuberculin skin test, carried out according to multi puncture method or Mantoux intradermal injection, are operating complexity and insufficient specificity. In fact, the test involves a first and a second patient visit for tuberculin injection and test reading by specialized sanitary staff, respectively. For the staff the contamination risk by intradermal injection syringes in case of TB-HIV co-infection occurs. As to the tuberculin test specificities, it is known that purified tuberculin protein derivative (Purified protein derivative, PPD) displays cross-reactivity with *M. bovis Bacillus Calmette-Guerin* (BCG) used for anti-tubercular vaccination and various environmental not tubercular mycobacteria, displaying high sequence homology with Kock *bacillus* genome (5, 6). Therefore, BCG vaccinated or also subjects recently in contact with MTB virulent species, i.e. as in research laboratories named, collection strain H37Rv (ATCC 27294), will be tuberculin test positive, even if with lower reaction intensity than MTB infected subjects. For this reason, positivity intensity limits discriminating MTB reactivity from that resulting from anti *M. bovis* BCG or non tubercular mycobacteria immunity, have been established.

Such intensity limits, however, are not sufficiently specific in order the tubercular infection in a vaccinated or environmental mycobacteria extensively exposed population to be diagnosed.

With respect to prevention of MTB infection the unique currently available vaccine for tubercular disease prophylaxis is *M. bovis Bacillus Calmette-Guerin* (BCG) (ATCC 27291), a vaccine based on an avirulent *M. bovis* strain, used all over the world from approximately 75 years.

In the light of above therefore it is apparent the need to provide for new diagnostic kits and vaccines based on specific peptide use suitable to overcome the disadvantages of up to now known art.

There are several studies correlating peripheral blood lymphocyte response to MTB proteins and peptides in the presence of latent infection, or recent contact with TB patients, and tubercular active disease using ELISPOT analysis for the detection of blood mononucleated cells suitable to produce IFN- γ as a result of stimulation (7-15). ELISPOT is a technique allowing, by stimulation of mononucleated blood cells on culture plate using sensitizing antibodies directed against cytokine (for example IFN- γ), the frequency of cytokine producing T lymphocytes as a response to stimulation with one or more antigens, which can be proteins, peptides or other target molecules, to be detected.

T lymphocytes recognize the antigen using the antigen T cell receptor (TCR) when the antigen is presented in peptide form (8-12 amino acid long), representing the epitope, bound to a molecule of histocompatibility major complex (MHC), a receptor family expressed on the plasmatic membrane of all the nucleated cells (as to class I MHC molecules) and antigen presenting cells like dendritic cells, macrophages etc. (as to class II MHC molecules). In humans MHC system is represented by various isotype variants, HLA-A, HLA-B and HLA-C for class I molecules; HLA-DP, HLA-DQ and HLA-DR for class II molecules. Each of said molecules displays a different number of allelic variants. Since T cell TCR recognized antigen repertoire is related to the ability of subject antigen presenting cell MHC receptors to bind peptides derived from the digestion of antigenic proteins, the indispensable condition in order the peptide to be recognised as antigen and therefore suitable to activate antigenic protein specific T lymphocytes is the susceptibility thereof to be bound by MHC receptors.

Genes encoding for HLA molecules are among most polymorphic genes occurring in human genome. It is noteworthy in this context the fact that many differences among individual allelic products of these molecules are variations of bases encoding for amino acid sequence modifications of regions involved in antigenic peptide binding. These sequence variations determine the binding properties of different allelic variants of HLA molecules and therefore the antigenic peptide repertoire with which said allelic variant will be able to form a tri-molecular complex together with T lymphocytes TCR and activate said lymphocytes.

In the context of MTB protein antigen recognizing, it is to be pointed out that every single mycobacterial epitope can be bound to one or more HLA allelic variants, but not necessarily all allelic variants expressed in a population. Moreover, since each not homozygote subject expresses at least two allelic variants of HLA-A, HLA-B, HLA-C, HLA-DP, HLA-DQ and two to four HLA-DR variants, in various subjects comprising the population under investigation different epitopes can be recognized in the context of the same or different allelic variants of different isotypes.

It is therefore apparent that the peptide set suitable to bind various allelic variants of HLA isotypes expressed by every

single subject in a population can be different. From above it results the need to use an antigenic peptide set suitable to represent in as exhaustive as possible way MTB peptide epitope repertoire recognized by T lymphocytes from subjects of the population under investigation. In this context it is to be pointed out that, although the whole MTB genome has been sequenced and is available to be studied, the antigens up to now used in immunological tests for tubercular infection diagnosis result from research about mycobacterium biochemical characterization during the in vitro growth step thereof in culture media and not from ex vivo experiments.

Patent application US2006/0115847 discloses an immunological diagnostic method for *M. tuberculosis* infection based on the combination of epitopes from proteins encoded by *M. tuberculosis* genome regions not occurring in BCG vaccine or most common not tubercular mycobacteria. The experimental part of said patent application reports results about various tested proteins, however the distribution of patient responses to single peptides is quite not homogeneous, in fact, as FIG. 1 shows, several TB patients are peptide insensitive. As to the patient response frequency to selected peptides, in table 6 it is shown that best individually tested peptide CFP10 induces a response in 10/15 patients, i.e. a 66.66% response frequency. Further, by means of combination of some peptides, as shown in table 7 of the US patent, sensitivity results of 92% for latent TB, i.e. PPD+ patients, 88% for active TB and 90% for TB patients under antibiotic treatment, have been obtained.

In prior studies, the authors of the present invention have identified a group of genes preferentially transcribed by human macrophages infecting MTB and characterized in that said genes belong to deletion regions of *M. bovis*-BCG vaccine species (WO 2005/021790).

The authors of the present invention now have analyzed proteins expressed by MTB in human macrophages, both in in vitro primary cultures, and or ex vivo in bronchoalveolar lavage (BAL) samples from active pulmonary TB patients. Using a software developed by the applicants allowing the ability of class II histocompatibility molecules to bind peptides of whole MTB genome to be analysed, some proteins, proved to be remarkably effective from the immunological point of view, have been selected. In summary, the study compared *M. tuberculosis* gene expression in three different growth environments: synthetic medium culture (Sauton's), monocyte-derived-human macrophage (MDM) infected in vitro with *M. tuberculosis*, alveolar macrophage (AM) from bronchoalveolar lavage (BAL) samples of pulmonary TB affected patients before the antibiotic therapy.

From thus obtained 9 gene groups, first 100 proteins, according to a combination criteria (modulation of expression, immunogenicity, tubercular Complex specificity, etc), have been selected. From these 100 proteins again a 30 protein group has been selected, for which a positive response in immunological tests on TB patients whole blood had been obtained (see table 1).

After an ulterior selection from 4 groups of subjects: Pulmonary TB before antibiotic therapy (n=13), recently exposed healthy contacts (TB patient relatives) PPD+(n=8); long term TB patient exposed healthy contacts (professional exposure of hospital workers) PPD+(n=5); BCG vaccinated negative controls, PPD- (n=4), firstly 43 peptides have been designed, synthesized and tested.

Then the 6 most sensible and specific peptides have been selected (see table 2), and the study has been repeated using an extended subject sample (see tables 3-5 and FIGS. 1-7).

The results obtained using said six peptides and a peptide belonging to ESAT6, i.e. an highly immunogenic protein occurring in both above mentioned commercial kits (FIG. 8), have been compared.

In summary, all the selected peptides displayed T-cell reactivity. Particularly, peptide #3 (SEQ ID NO:71 of the present invention) displays an elevated sensitivity comparable, if not more elevated, to multi-epitope ESAT-6 protein derived control peptide. It is noteworthy that, as in panel estimated (i.e. by estimating ex-post total data of each individual peptide), 6 multi-epitope peptides are recognized by about 75% of the subjects with active TB (a result comparable, in this series, to Quantiferon-TB gold in-Tube, as shown in Table 4). Data are perfectly similar to the result obtained in a patient subgroup tested using said 6 peptides concurrently in the same well (as shown in Table 5).

Peptide optimal diagnostic sensitivity is associated together with an optimal specificity. In fact, the reported peptide response is limited to active TB subjects, recent exposure contacts and exposed sanitary staff (as shown in Table 4 and FIG. 9). It is noteworthy that, although the data must be supported by larger subject number, no peptide response from *M. bovis* BCG antitubercular vaccinated control subjects, not even from Quantiferon-TB gold in-Tube positive 3 subjects, individually has been detected.

Further, the peptides allow the commercial test sensitivity, i.e. current gold reference standard for tubercular infection diagnosis (FIG. 10), to be enhanced. Both when panel estimated and directly tested within same well, 6 selected peptides allow Quantiferon-TB gold in-Tube to be enhanced from 75% to 89% (+14%) and from 71% to 83% (+13%), respectively, in subjects with active TB (see Table 4 and 5).

It is therefore a specific object of the present invention the use of at least 6 peptides, derived from *Mycobacterium tuberculosis* and comprising at least one T-cell epitope, in association with ESAT6 and CFP10 and, optionally with TB7.7, as biomarkers in an in vitro test for the detection of *Mycobacterium tuberculosis* infection in a subject, said peptides being chosen from the group consisting of: TAWITAWPGLMV (SEQ ID NO:24), ELMARAAVLGSAH (SEQ ID NO:21), RPVRRVLLFWPSSGPAP (SEQ ID NO:70), GSVRQLPSVLKPPLITLRTLTLTSLG (SEQ ID NO:71), GEIIFISGRNLGaa (SEQ ID NO:13), AVIVRSELLTQYL (SEQ ID NO:22), LAWITAWPGLMV (SEQ ID NO:85), GEIIFISGRNLG (SEQ ID NO:86) or SALLRRLSTCPPES (SEQ ID NO:87). According to an embodiment of the present invention the peptides are the following six peptides: ELMARAAVLGSAH (SEQ ID NO:21), RPVRRVLLFWPSSGPAP (SEQ ID NO:70), GSVRQLPSVLKPPLITLRTLTLTSLG (SEQ ID NO:71), AVIVRSELLTQYL (SEQ ID NO:22), TAWITAWPGLMV (SEQ ID NO:24) and GEIIFISGRNLGaa (SEQ ID NO:13). According to a further embodiment of the present invention, the peptides are the following six peptides: ELMARAAVLGSAH (SEQ ID NO:21), RPVRRVLLFWPSSGPAP (SEQ ID NO:70), GSVRQLPSVLKPPLITLRTLTLTSLG (SEQ ID NO:71), AVIVRSELLTQYL (SEQ ID NO:22), LAWITAWPGLMV (SEQ ID NO:85) and GEIIFISGRNLG (SEQ ID NO:86). According to another embodiment of the present invention the peptides are all the following nine peptides: TAWITAWPGLMV (SEQ ID NO:24), ELMARAAVLGSAH (SEQ ID NO:21), RPVRRVLLFWPSSGPAP (SEQ ID NO:70), GSVRQLPSVLKPPLITLRTLTLTSLG (SEQ ID NO:71), GEIIFISGRNLGaa (SEQ ID NO:13), AVIVRSELLTQYL (SEQ ID NO:22), LAWITAWPGLMV (SEQ ID NO:85), GEIIFISGRNLG (SEQ ID NO:86) and SALLRRLSTCPPES (SEQ ID NO:87).

The present invention concerns also the use of at least one peptide, derived from *Mycobacterium tuberculosis* and comprising at least one T-cell epitope, in association with ESAT6 and CFP10 and, optionally, with TB7.7, as biomarker in an in vitro test for the detection of *Mycobacterium tuberculosis* infection in a subject, said peptide being chosen from the group consisting of: TAWITAWPGLMV (SEQ ID NO:24), ELMARAAVLGSAH (SEQ ID NO:21), RPVRRVLLFWPSSGPAP (SEQ ID NO:70), GSVRQLPSVLKPPLITLRTLTLTSLG (SEQ ID NO:71), GEIIFISGRNLGaa (SEQ ID NO:13), AVIVRSELLTQYL (SEQ ID NO:22), LAWITAWPGLMV (SEQ ID NO:85), GEIIFISGRNLG (SEQ ID NO:86) or SALLRRLSTCPPES (SEQ ID NO:87). Particularly said at least one peptide can be LAWITAWPGLMV (SEQ ID NO:85) or TAWITAWPGLMV (SEQ ID NO:24).

It is further object of the present invention a method for in vitro diagnosing infection by a *Mycobacterium tuberculosis* in a subject,

said method comprising incubating a blood sample comprising lymphocytes from said subject in the presence of at least six peptides, derived from *Mycobacterium tuberculosis* and comprising at least one T-cell epitope, in association with ESAT6 and CFP10 and, optionally with TB7.7,

for a time and under conditions sufficient to stimulate the lymphocytes to produce an effector molecule, wherein the presence or level of the effector molecule is indicative of the lymphocytes derived from a subject infected with or prior exposed to the *Mycobacterium* species,

said at least six peptides being chosen from the group consisting of TAWITAWPGLMV (SEQ ID NO:24), ELMARAAVLGSAH (SEQ ID NO:21), RPVRRVLLFWPSSGPAP (SEQ ID NO:70), GSVRQLPSVLKPPLITLRTLTLTSLG (SEQ ID NO:71), GEIIFISGRNLGaa (SEQ ID NO:13), AVIVRSELLTQYL (SEQ ID NO:22), LAWITAWPGLMV (SEQ ID NO:85), GEIIFISGRNLG (SEQ ID NO:86) or SALLRRLSTCPPES (SEQ ID NO:87).

According to an embodiment of the method of the present invention, the peptides are the following six peptides: ELMARAAVLGSAH (SEQ ID NO:21), RPVRRVLLFWPSSGPAP (SEQ ID NO:70), GSVRQLPSVLKPPLITLRTLTLTSLG (SEQ ID NO:71), AVIVRSELLTQYL (SEQ ID NO:22), TAWITAWPGLMV (SEQ ID NO:24) and GEIIFISGRNLGaa (SEQ ID NO:13). According to a further embodiment of the method of the present invention, the peptides are the following six peptides: ELMARAAVLGSAH (SEQ ID NO:21), RPVRRVLLFWPSSGPAP (SEQ ID NO:70), GSVRQLPSVLKPPLITLRTLTLTSLG (SEQ ID NO:71), AVIVRSELLTQYL (SEQ ID NO:22), LAWITAWPGLMV (SEQ ID NO:85) and GEIIFISGRNLG (SEQ ID NO:86).

According to another embodiment, the peptides are all the following nine peptides: TAWITAWPGLMV (SEQ ID NO:24), ELMARAAVLGSAH (SEQ ID NO:21), RPVRRVLLFWPSSGPAP (SEQ ID NO:70), GSVRQLPSVLKPPLITLRTLTLTSLG (SEQ ID NO:71), GEIIFISGRNLGaa (SEQ ID NO:13), AVIVRSELLTQYL (SEQ ID NO:22), LAWITAWPGLMV (SEQ ID NO:85), GEIIFISGRNLG (SEQ ID NO:86) and SALLRRLSTCPPES (SEQ ID NO:87).

It is an object of the present invention a method for in vitro diagnosing infection by a *Mycobacterium tuberculosis* in a subject,

said method comprising incubating a blood sample comprising lymphocytes from said subject in the presence of at least one peptide, derived from *Mycobacterium tuberculosis* and comprising at least one T-cell epitope, in association with ESAT6 and CFP10 and, optionally, with TB7.7,

for a time and under conditions sufficient to stimulate the lymphocytes to produce an effector molecule, wherein the

presence or level of the effector molecule is indicative of the lymphocytes derived from a subject infected with or prior exposed to the *Mycobacterium* species,

said at least one peptide being chosen from the group consisting of TAWITAWPGLMV (SEQ ID NO:24), ELMARA-RAAVLGSAAH (SEQ ID NO:21), RPVRRVLLFWPSSGPAP (SEQ ID NO:70), GSVRQLPSVLKPLITLRTLTLG (SEQ ID NO:71), GEIIFISGRLNGaa (SEQ ID NO:13), AVIVRSELLTQYL (SEQ ID NO:22), LAWITAWPGLMV (SEQ ID NO:85), GEIIFISGRLNG (SEQ ID NO:86) or SALLRRLSTCPPES (SEQ ID NO:87). For example, said at least one peptide can be LAWITAWPGLMV (SEQ ID NO:85) or TAWITAWPGLMV (SEQ ID NO:24).

It is further object of the present invention a method for the in vitro diagnosis of infection by *Mycobacterium tuberculosis* in a subject, said method including incubating a blood sample comprising lymphocytes from the subject with ESAT6 and CFP10, and optionally with TB7.7, and measuring release of interferon- γ by the lymphocytes, said method being characterized in that the incubation is carried out further in the presence of at least six peptides chosen from the group consisting of TAWITAWPGLMV (SEQ ID NO:24), ELMARA-RAAVLGSAAH (SEQ ID NO:21), RPVRRVLLFWPSSGPAP (SEQ ID NO:70), GSVRQLPSVLKPLITLRTLTLG (SEQ ID NO:71), GEIIFISGRLNGaa (SEQ ID NO:13), AVIVRSELLTQYL (SEQ ID NO:22), LAWITAWPGLMV (SEQ ID NO:85), GEIIFISGRLNG (SEQ ID NO:86) or SALLRRLSTCPPES (SEQ ID NO:87),

wherein the level of sensitivity and/or selectivity for the detection of *Mycobacterium tuberculosis* is higher compared to the sensitivity and/or selected using ESAT6 and CFP10, optionally with TB7.7. According to an embodiment of the present invention, the peptides are the following six peptides: ELMARA-RAAVLGSAAH (SEQ ID NO:21), RPVRRVLLFWPSSGPAP (SEQ ID NO:70), GSVRQLPSVLKPLITLRTLTLG (SEQ ID NO:71), AVIVRSELLTQYL (SEQ ID NO:22), TAWITAWPGLMV (SEQ ID NO:24) and GEIIFISGRLNGaa (SEQ ID NO:13). According to a further embodiment the peptides are the following six peptides: ELMARA-RAAVLGSAAH (SEQ ID NO:21), RPVRRVLLFWPSSGPAP (SEQ ID NO:70), GSVRQLPSVLKPLITLRTLTLG (SEQ ID NO:71), AVIVRSELLTQYL (SEQ ID NO:22), LAWITAWPGLMV (SEQ ID NO:85) and GEIIFISGRLNG (SEQ ID NO:86). According to another embodiment the peptides are all the following nine peptides: TAWITAWPGLMV (SEQ ID NO:24), ELMARA-RAAVLGSAAH (SEQ ID NO:21), RPVRRVLLFWPSSGPAP (SEQ ID NO:70), GSVRQLPSVLKPLITLRTLTLG (SEQ ID NO:71), GEIIFISGRLNGaa (SEQ ID NO:13), AVIVRSELLTQYL (SEQ ID NO:22), LAWITAWPGLMV (SEQ ID NO:85), GEIIFISGRLNG (SEQ ID NO:86) and SALLRRLSTCPPES (SEQ ID NO:87).

The present invention concerns also a method for the in vitro diagnosis of infection by *Mycobacterium tuberculosis* in a subject, said method including incubating a blood sample comprising lymphocytes from the subject with ESAT6 and CFP10, and optionally with TB7.7, and measuring release of interferon- γ by the lymphocytes, said method being characterized in that the incubation is carried out further in the presence of at least one peptide chosen from the group consisting of TAWITAWPGLMV (SEQ ID NO:24), ELMARA-RAAVLGSAAH (SEQ ID NO:21), RPVRRVLLFWPSSGPAP (SEQ ID NO:70), GSVRQLPSVLKPLITLRTLTLG (SEQ ID NO:71), GEIIFISGRLNGaa (SEQ ID NO:13), AVIVRSELLTQYL (SEQ ID NO:22), LAWITAWPGLMV (SEQ ID NO:85), GEIIFISGRLNG (SEQ ID NO:86) or SALLRRLSTCPPES (SEQ ID NO:87).

wherein the level of sensitivity and/or selectivity for the detection of *Mycobacterium tuberculosis* is higher compared to the sensitivity and/or selected using ESAT6 and CFP10, optionally with TB7.7. Said at least one peptide can be LAWITAWPGLMV (SEQ ID NO:85) or TAWITAWPGLMV (SEQ ID NO:24).

It is further object of the present invention the use of at least one biomarker selected from the list consisting of:

(i) a protein selected from Rv0023, Rv0182c, Rv0290, Rv0601c, Rv0647c, Rv0724A, Rv0890c, Rv1251c, Rv1398c, Rv1478, Rv1497, Rv1575, Rv1578c, Rv1899c, Rv2137c, Rv2333c, Rv2548, Rv2557, Rv2816c, Rv2990, Rv3094c, Rv3107c, Rv3188, Rv3239c, Rv3296, Rv3425, Rv3446c, Rv3479, Rv3482c, Rv3780, derived from a *Mycobacterium* species or related organism and comprising at least one T-cell epitope;

(ii) an homolog of the protein as defined in (i) having an amino acid sequence with at least 80% similarity in comparison to one of said protein after optimal alignment; and

(iii) a peptide fragment of the protein as defined in (i) or (ii) having a T-cell epitope or a chemical analog thereof; in an in vitro test for the detection of *Mycobacterium* infection in a subject, i.e. an human or non human animal subject.

The *Mycobacterium* species can be selected from *M. tuberculosis*, *M. bovis*, *M. bovis* BCG, *M. africanum*, *M. canetti*, *M. caprae*, *M. microti*, *M. pinnipedii*, *M. avium*, *M. avium paratuberculosis*, *M. avium silvaticum*, *M. avium* "hominis-suis", *M. colombiense*, *M. asiaticum*, *M. gordonae*, *M. gastri*, *M. kansasii*, *M. hiberniae*, *M. nonchromogenicum*, *M. terrae*, *M. triviale*, *M. ulcerans*, *M. pseudoshottsii*, *M. shottsii*, *M. triplex*, *M. genavense*, *M. florentinum*, *M. lentiflavum*, *M. palustre*, *M. kubicae*, *M. parascrofulaceum*, *M. heidelbergense*, *M. interjectum*, *M. simiae*, *M. branderi*, *M. cookii*, *M. celatum*, *M. bohemicum*, *M. haemophilum*, *M. malmoense*, *M. szulgai*, *M. leprae*, *M. lepraemurium*, *M. lepromatosis*, *M. africanum*, *M. botniense*, *M. chimaera*, *M. conspicuum*, *M. doricum*, *M. farcinogenes*, *M. heckeshornense*, *M. intracellulare*, *M. lacus*, *M. marinum*, *M. monacense*, *M. montefiorensense*, *M. murale*, *M. nebraskense*, *M. saskatchewanense*, *M. scrofulaceum*, *M. shimoidei*, *M. tusciae*, *M. xenopi*, *M. intermedium*, *M. abscessus*, *M. chelonae*, *M. bolletii*, *M. fortuitum*, *M. fortuitum* subsp. *acetamidolyticum*, *M. boenickei*, *M. peregrinum*, *M. porcinum*, *M. senegalense*, *M. septicum*, *M. neworleansense*, *M. houstonense*, *M. mucogenicum*, *M. mag-eritense*, *M. brisbanense*, *M. cosmeticum*, *M. parafortuitum*, *M. austroafricanum*, *M. diernhoferi*, *M. hodleri*, *M. neoaurum*, *M. frederiksbergense*, *M. aurum*, *M. vaccae*, *M. chitae*, *M. fallax*, *M. confluentis*, *M. flavescens*, *M. madagascariense*, *M. phlei*, *M. smegmatis*, *M. goodii*, *M. wolinskyi*, *M. thermoresistibile*, *M. gadium*, *M. komossense*, *M. obuense*, *M. sphagni*, *M. agri*, *M. aichiense*, *M. alvei*, *M. arupense*, *M. brumae*, *M. canariense*, *M. chubuense*, *M. conceptionense*, *M. duvalii*, *M. elephantis*, *M. gilvum*, *M. hassiacum*, *M. hol-saticum*, *M. immunogenum*, *M. massiliense*, *M. moriokaense*, *M. psychrotolerans*, *M. pyrenivorans*, *M. vanbaalenii*, *M. pulveris*, *M. arosiense*, *M. aubagnense*, *M. caprae*, *M. chlorophenicum*, *M. fluoroanthenvivorans*, *M. kumamotoense*, *M. novocastrense*, *M. parmense*, *M. phocaicum*, *M. poriferae*, *M. rhodesiae*, *M. seoulense* and *M. tokaiense*. Preferably, the *Mycobacterium* species is *Mycobacterium tuberculosis*.

According to the above use, the peptide fragment as defined in (iii) can comprise or consist of an amino acid sequence selected from TAWITAWPGLMV (SEQ ID NO:24), AVIVRSELLTQYL (SEQ ID NO:22), GSVRQLPSVLKPLITLRTLTLG (SEQ ID NO:71), RPVRRVLLFWPSSGPAP (SEQ ID NO:70), GEIIFISGRL-

NGaa (SEQ ID NO:13), ELMARAAVLGSAH (SEQ ID NO:21), LAWITAWPGLMV (SEQ ID NO:85), GEIIFISGRLNG (SEQ ID NO:86), SALLRRLSTCPPES (SEQ ID NO:87).

The above use can further comprise the use of one or more *Mycobacterium* protein or peptide fragment thereof or chemical analog derived therefrom selected from ESAT6, CFP10, TB7.7 and PPD.

It is further object of the present invention the use of at least one biomarker selected from the list consisting of:

(i) a protein selected from Rv0023, Rv0182c, Rv0290, Rv0601c, Rv0647c, Rv0724A, Rv0890c, Rv1251c, Rv1398c, Rv1478, Rv1497, Rv1575, Rv1578c, Rv1899c, Rv2137c, Rv2333c, Rv2548, Rv2557, Rv2816c, Rv2990, Rv3094c, Rv3107c, Rv3188, Rv3239c, Rv3296, Rv3425, Rv3446c, Rv3479, Rv3482c, Rv3780, derived from a *Mycobacterium* species or related organism and comprising at least one T-cell epitope;

(ii) an homolog of the protein as defined in (i) having an amino acid sequence with at least 80% similarity to one of said protein after optimal alignment; and

(iii) a peptide fragment of the protein as defined in (i) or (ii) having a T-cell epitope or a chemical analog thereof; and

(iv) a *Mycobacterium* derived protein or fragment thereof or chemical analog thereof selected from the list consisting of:

- (a) ESAT;
- (b) CFP10;
- (c) TB7.7; and
- (d) PPD;

in an in vitro test for the detection of *Mycobacterium* infection in a subject i.e. an human or a non-human animal subject.

The *Mycobacterium* species can be selected from *M. tuberculosis*, *M. bovis*, *M. bovis* BCG, *M. africanum*, *M. canetti*, *M. caprae*, *M. microti*, *M. pinnipedii*, *M. avium*, *M. avium paratuberculosis*, *M. avium silvaticum*, *M. avium* "hominis-suis", *M. colombiense*, *M. asiaticum*, *M. gordonae*, *M. gastri*, *M. kansasii*, *M. hiberniae*, *M. nonchromogenicum*, *M. terrae*, *M. triviale*, *M. ulcerans*, *M. pseudoshottsii*, *M. shottsii*, *M. triplex*, *M. genavense*, *M. florentinum*, *M. lentiflavum*, *M. palustre*, *M. kubicae*, *M. parascrofulaceum*, *M. heidelbergense*, *M. interjectum*, *M. simiae*, *M. branderi*, *M. cookii*, *M. celatum*, *M. bohemicum*, *M. haemophilum*, *M. malmoense*, *M. szulgai*, *M. leprae*, *M. lepraemurium*, *M. lepromatosis*, *M. africanum*, *M. botniense*, *M. chimaera*, *M. conspicuum*, *M. doricum*, *M. farcinogenes*, *M. heckeshornense*, *M. intracellulare*, *M. lacus*, *M. marinum*, *M. monacense*, *M. montefiorensense*, *M. murale*, *M. nebraskense*, *M. saskatchewanense*, *M. scrofulaceum*, *M. shimoidei*, *M. tusciae*, *M. xenopi*, *M. intermedium*, *M. abscessus*, *M. chelonae*, *M. bolletii*, *M. fortuitum*, *M. fortuitum* subsp. *acetamidolyticum*, *M. boenickei*, *M. peregrinum*, *M. porcinum*, *M. senegalense*, *M. septicum*, *M. neworleansense*, *M. houstonense*, *M. mucogenicum*, *M. mag-eritense*, *M. brisbanense*, *M. cosmeticum*, *M. parafortuitum*, *M. austroafricanum*, *M. diernhoferi*, *M. hodleri*, *M. neoaurum*, *M. frederiksbergense*, *M. aurum*, *M. vaccae*, *M. chitae*, *M. fallax*, *M. confluentis*, *M. flavescens*, *M. madagascariense*, *M. phlei*, *M. smegmatis*, *M. goodii*, *M. wolinskyi*, *M. thermoresistibile*, *M. gadium*, *M. komossense*, *M. obuense*, *M. sphagni*, *M. agri*, *M. aichiense*, *M. alvei*, *M. arupense*, *M. brumae*, *M. canariasense*, *M. chubuense*, *M. conceptionense*, *M. duvalii*, *M. elephantis*, *M. gilvum*, *M. hassiacum*, *M. hol-saticum*, *M. immunogenum*, *M. massiliense*, *M. moriokaense*, *M. psychrotolerans*, *M. pyrenivorans*, *M. vanbaalenii*, *M. pulveris*, *M. arosiense*, *M. aubagnense*, *M. caprae*, *M. chlo-phenolicum*, *M. fluoroanthenivorans*, *M. kumamotonense*, *M. novocastrense*, *M. parmense*, *M. phocaicum*, *M.*

poriferae, *M. rhodesiae*, *M. seoulense* and *M. tokaiense*. Preferably, the *Mycobacterium* species is *Mycobacterium tuberculosis*.

According to the above use, the peptide fragment as defined in (iii) can comprise or consist of an amino acid sequence selected from TAWITAWPGLMV (SEQ ID NO:24), AVIVRSELLTQYL (SEQ ID NO:22), GSVRQLPSVLKPLTLRLLTSLG (SEQ ID NO:71), RPVRRVLLFWPSSGPAP (SEQ ID NO:70), GEIIFISGRL-NGaa (SEQ ID NO:13), ELMARAAVLGSAH (SEQ ID NO:21), LAWITAWPGLMV (SEQ ID NO:85), GEIIFISGRLNG (SEQ ID NO:86), SALLRRLSTCPPES (SEQ ID NO:87).

It is a further object of the present invention the use of at least one nucleic acid molecule encoding for a biomarker selected from the list consisting of:

(i) a protein selected from Rv0023, Rv0182c, Rv0290, Rv0601c, Rv0647c, Rv0724A, Rv0890c, Rv1251c, Rv1398c, Rv1478, Rv1497, Rv1575, Rv1578c, Rv1899c, Rv2137c, Rv2333c, Rv2548, Rv2557, Rv2816c, Rv2990, Rv3094c, Rv3107c, Rv3188, Rv3239c, Rv3296, Rv3425, Rv3446c, Rv3479, Rv3482c, Rv3780, derived from a *Mycobacterium* species or related organism and comprising at least one T-cell epitope;

(ii) an homolog of the protein as defined in (i) having an amino acid sequence with at least 80% similarity to one of said protein after optimal alignment; and

(iii) a peptide fragment of the protein as defined in (i) or (ii) having a T-cell epitope or chemical analog thereof;

in an in vitro test for the detection of *Mycobacterium* infection in a subject i.e. an human or a non-human animal subject.

The *Mycobacterium* species is selected from *M. tuberculosis*, *M. bovis*, *M. bovis* BCG, *M. africanum*, *M. canetti*, *M. caprae*, *M. microti*, *M. pinnipedii*, *M. avium*, *M. avium paratuberculosis*, *M. avium silvaticum*, *M. avium* "hominis-suis", *M. colombiense*, *M. asiaticum*, *M. gordonae*, *M. gastri*, *M. kansasii*, *M. hiberniae*, *M. nonchromogenicum*, *M. terrae*, *M. triviale*, *M. ulcerans*, *M. pseudoshottsii*, *M. shottsii*, *M. triplex*, *M. genavense*, *M. florentinum*, *M. lentiflavum*, *M. palustre*, *M. kubicae*, *M. parascrofulaceum*, *M. heidelbergense*, *M. interjectum*, *M. simiae*, *M. branderi*, *M. cookii*, *M. celatum*, *M. bohemicum*, *M. haemophilum*, *M. malmoense*, *M. szulgai*, *M. leprae*, *M. lepraemurium*, *M. lepromatosis*, *M. africanum*, *M. botniense*, *M. chimaera*, *M. conspicuum*, *M. doricum*, *M. farcinogenes*, *M. heckeshornense*, *M. intracellulare*, *M. lacus*, *M. marinum*, *M. monacense*, *M. montefiorensense*, *M. murale*, *M. nebraskense*, *M. saskatchewanense*, *M. scrofulaceum*, *M. shimoidei*, *M. tusciae*, *M. xenopi*, *M. intermedium*, *M. abscessus*, *M. chelonae*, *M. bolletii*, *M. fortuitum*, *M. fortuitum* subsp. *acetamidolyticum*, *M. boenickei*, *M. peregrinum*, *M. porcinum*, *M. senegalense*, *M. septicum*, *M. neworleansense*, *M. houstonense*; *M. mucogenicum*, *M. mag-eritense*, *M. brisbanense*, *M. cosmeticum*, *M. parafortuitum*, *M. austroafricanum*, *M. diernhoferi*, *M. hodleri*, *M. neoaurum*, *M. frederiksbergense*, *M. aurum*, *M. vaccae*, *M. chitae*, *M. fallax*, *M. confluentis*, *M. flavescens*, *M. madagascariense*, *M. phlei*, *M. smegmatis*, *M. goodii*, *M. wolinskyi*, *M. thermoresistibile*, *M. gadium*, *M. komossense*, *M. obuense*, *M. sphagni*, *M. agri*, *M. aichiense*, *M. alvei*, *M. arupense*, *M. brumae*, *M. canariasense*, *M. chubuense*, *M. conceptionense*, *M. duvalii*, *M. elephantis*, *M. gilvum*, *M. hassiacum*, *M. hol-saticum*, *M. immunogenum*, *M. massiliense*, *M. moriokaense*, *M. psychrotolerans*, *M. pyrenivorans*, *M. vanbaalenii*, *M. pulveris*, *M. arosiense*, *M. aubagnense*, *M. caprae*, *M. chlo-phenolicum*, *M. fluoroanthenivorans*, *M. kumamotonense*, *M. novocastrense*, *M. parmense*, *M. phocaicum*, *M.*

poriferae, *M. rhodesiae*, *M. seoulense* and *M. tokaiense*. Preferably, the *Mycobacterium* species is *Mycobacterium tuberculosis*.

According to the above use, the peptide fragment in (iii) can comprise or consist of an amino acid sequence selected from TAWITAWPGLMV (SEQ ID NO:24), AVIVRSELLTQYL (SEQ ID NO:22), GSVRQLPSVLKPPLITLRTLTLG (SEQ ID NO:71), RPVRRVLLFWPSSGPAP (SEQ ID NO:70), GEIIFISGRLNGaa (SEQ ID NO:13), ELMARA AVLGSAH (SEQ ID NO:21), LAWITAWPGLMV (SEQ ID NO:85), GEIIFISGRLNG (SEQ ID NO:86), SALLRRLSTCPPES (SEQ ID NO:87). Said use can further comprise use of a nucleic acid molecule encoding for a *Mycobacterium* protein or peptide fragment derived therefrom selected from ESAT6, CFP10, TB7.7 and PPD or an homolog thereof.

It is further object of the present invention, an isolated protein selected from the list consisting of Rv0023, Rv0182c, Rv0290, Rv0601c, Rv0647c, Rv0724A, Rv0890c, Rv1251c, Rv1398c, Rv1478, Rv1497, Rv1575, Rv1578c, Rv1899c, Rv2137c, Rv2333c, Rv2548, Rv2557, Rv2816c, Rv2990, Rv3094c, Rv3107c, Rv3188, Rv3239c, Rv3296, Rv3425, Rv3446c, Rv3479, Rv3482c, Rv3780, derived from a *Mycobacterium* species and comprising a T-cell epitope. The isolated peptide of the protein according to the invention can comprise a T-cell epitope or a chemical analog thereof. The isolated peptide according can comprise or consist of an amino acid sequence selected from TAWITAWPGLMV (SEQ ID NO:24), AVIVRSELLTQYL (SEQ ID NO:22), GSVRQLPSVLKPPLITLRTLTLG (SEQ ID NO:71), RPVRRVLLFWPSSGPAP (SEQ ID NO:70), GEIIFISGRLNGaa (SEQ ID NO:13), ELMARA AVLGSAH (SEQ ID NO:21), LAWITAWPGLMV (SEQ ID NO:85), GEIIFISGRLNG (SEQ ID NO:86), SALLRRLSTCPPES (SEQ ID NO:87).

It is further object of the present invention an isolated nucleic acid molecule encoding for the above mentioned protein or peptide according to the present invention.

The present invention concerns a vector comprising the above mentioned nucleic acid molecule, and isolated cell comprising said vector.

It is a further object of the present invention a kit comprising a container, said container comprising the above mentioned at least one protein or at least one a peptide or at least one a nucleic acid molecule.

The present invention concerns a method for in vitro diagnosing infection by a *Mycobacterium* species in a subject, said method comprising incubating a blood sample comprising lymphocytes from said subject in the presence of at least one biomarker selected from the list consisting of:

(i) a protein selected from Rv0023, Rv0182c, Rv0290, Rv0601c, Rv0647c, Rv0724A, Rv0890c, Rv1251c, Rv1398c, Rv1478, Rv1497, Rv1575, Rv1578c, Rv1899c, Rv2137c, Rv2333c, Rv2548, Rv2557, Rv2816c, Rv2990, Rv3094c, Rv3107c, Rv3188, Rv3239c, Rv3296, Rv3425, Rv3446c, Rv3479, Rv3482c, Rv3780, derived from a *Mycobacterium* species or related organism and comprising at least one T-cell epitope;

(ii) an homolog of the protein as defined in (i) having an amino acid sequence with at least 80% similarity to one of said protein after optimal alignment; and

(iii) a peptide fragment of the protein as defined in (i) or (ii) having a T-cell epitope or a chemical analog thereof; for a time and under conditions sufficient to stimulate the lymphocytes to produce an effector molecule, wherein the presence or level of the effector molecule is indicative of the lymphocytes derived from a subject infected with or prior

exposed to the *Mycobacterium* species. The subject can be an human or a non-human animal. The incubation between blood and biomarker can occur in a test-tube, optionally in the presence of heparin, in the presence of an added carbohydrate. According to an embodiment of the present invention, the incubation can occur further in the presence of a *Mycobacterium* protein or peptide fragment thereof or chemical analog thereof derived therefrom selected from ESAT6, CFP10, TB7.7 and PPD or mixture thereof. The *Mycobacterium* species is selected from *M. tuberculosis*, *M. bovis*, *M. bovis* BCG, *M. africanum*, *M. canetti*, *M. caprae*, *M. microti*, *M. pinnipedii*, *M. avium*, *M. avium paratuberculosis*, *M. avium silvaticum*, *M. avium* "hominissuis", *M. colombiense*, *M. asiaticum*, *M. gordonae*, *M. gastri*, *M. kansasii*, *M. hiberniae*, *M. nonchromogenicum*, *M. terrae*, *M. triviale*, *M. ulcerans*, *M. pseudoshottsii*, *M. shottsii*, *M. triplex*, *M. genavense*, *M. florentinum*, *M. lentiflavum*, *M. palustre*, *M. kubicae*, *M. parascrofulaceum*, *M. heidelbergense*, *M. interjectum*, *M. simiae*, *M. branderi*, *M. cookii*, *M. celatum*, *M. bohemicum*, *M. haemophilum*, *M. malmoeense*, *M. szulgai*, *M. leprae*, *M. lepraemurium*, *M. lepromatosis*, *M. africanum*, *M. botniense*, *M. chimaera*, *M. conspicuum*, *M. doricum*, *M. farcinogenes*, *M. heckeshornense*, *M. intracellulare*, *M. lacus*, *M. marinum*, *M. monacense*, *M. montefiorensense*, *M. murale*, *M. nebraskense*, *M. saskatchewanense*, *M. scrofulaceum*, *M. shimoidei*, *M. tusciae*, *M. xenopi*, *M. intermedium*, *M. abscessus*, *M. chelonae*, *M. bolletii*, *M. fortuitum*, *M. fortuitum* subsp. *acetamidolyticum*, *M. boenickei*, *M. peregrinum*, *M. porcinum*, *M. senegalense*, *M. septicum*, *M. neworleansense*, *M. houstonense*, *M. mucogenicum*, *M. mageritense*, *M. brisbanense*, *M. cosmeticum*, *M. parafortuitum*, *M. austroafricanum*, *M. diernhoferi*, *M. hodleri*, *M. neoaurum*, *M. frederiksbergense*, *M. aurum*, *M. vaccae*, *M. chitae*, *M. fallax*, *M. confluentis*, *M. flavescens*, *M. madagascariense*, *M. phlei*, *M. smegmatis*, *M. goodii*, *M. wolinskyi*, *M. thermoresistibile*, *M. gadium*, *M. komossense*, *M. obuense*, *M. sphagni*, *M. agri*, *M. aichiense*, *M. alvei*, *M. arupense*, *M. brumae*, *M. canariasense*, *M. chubuense*, *M. conceptionense*, *M. duvalii*, *M. elephantis*, *M. gilvum*, *M. hassiacum*, *M. holsaticum*, *M. immunogenum*, *M. massiliense*, *M. moriokaense*, *M. psychrotolerans*, *M. pyrenivorans*, *M. vanbaalenii*, *M. pulveris*, *M. arosiense*, *M. aubagnense*, *M. caprae*, *M. chlorophenolicum*, *M. fluoroanthenvivorans*, *M. kumamotoense*, *M. novocastrense*, *M. parmense*, *M. phocaicum*, *M. poriferae*, *M. rhodesiae*, *M. seoulense* and *M. tokaiense*. Preferably, the *Mycobacterium* species is *Mycobacterium tuberculosis*.

According to the above-mentioned method of the invention, the peptide as defined in (ii) comprises or consists of an amino acid sequence selected from TAWITAWPGLMV (SEQ ID NO:24), AVIVRSELLTQYL (SEQ ID NO:22), GSVRQLPSVLKPPLITLRTLTLG (SEQ ID NO:71), RPVRRVLLFWPSSGPAP (SEQ ID NO:70), GEIIFISGRLNGaa (SEQ ID NO:13), ELMARA AVLGSAH (SEQ ID NO:21), LAWITAWPGLMV (SEQ ID NO:85), GEIIFISGRLNG (SEQ ID NO:86), SALLRRLSTCPPES (SEQ ID NO:87). The effector molecule can be selected from interferon- γ , a cytokine, an interleukin and TNF- α , preferably interferon- γ .

It is a further object of the present invention an isolated antibody specific for the protein or peptide defined above according.

The invention concerns also a method for the in vitro diagnosis of infection by *Mycobacterium tuberculosis* in a subject, said method including incubating a blood sample comprising lymphocytes from the subject with one or more of ESAT6, CFP10, TB7.7 and/or PPD and measuring release of interferon- γ by the lymphocytes, said method being charac-

terized in that the incubation is carried out further in the presence of at least one biomarker selected from:

(i) a protein selected from Rv0023, Rv0182c, Rv0290, Rv0601c, Rv0647c, Rv0724A, Rv0890c, Rv1251c, Rv1398c, Rv1478, Rv1497, Rv1575, Rv1578c, Rv1899c, Rv2137c, Rv2333c, Rv2548, Rv2557, Rv2816c, Rv2990, Rv3094c, Rv3107c, Rv3188, Rv3239c, Rv3296, Rv3425, Rv3446c, Rv3479, Rv3482c, Rv3780, derived from a *Mycobacterium* species or related organism and comprising at least one T-cell epitope;

(ii) an homolog of the protein as defined in (i) having an amino acid sequence with at least 80% similarity to one of said protein after optimal alignment; and

(iii) a peptide fragment of the protein as defined in (i) or (ii) having a T-cell epitope or chemical analog thereof; wherein the level of sensitivity and/or selectivity for the detection of *Mycobacterium tuberculosis* is higher compared to the sensitivity and/or selected using one or more of ESAT6, CFP10, TB7.7 and/or PPD alone.

In addition, the present invention concerns vaccine for the treatment or prophylaxis of infection by a *Mycobacterium* species, said vaccine comprising or consisting of at least one agent selected from the list consisting of:

(i) a protein selected from Rv0023, Rv0182c, Rv0290, Rv0601c, Rv0647c, Rv0724A, Rv0890c, Rv1251c, Rv1398c, Rv1478, Rv1497, Rv1575, Rv1578c, Rv1899c, Rv2137c, Rv2333c, Rv2548, Rv2557, Rv2816c, Rv2990, Rv3094c, Rv3107c, Rv3188, Rv3239c, Rv3296, Rv3425, Rv3446c, Rv3479, Rv3482c, Rv3780, derived from a *Mycobacterium* species or related organism and comprising at least one T-cell epitope;

(ii) an homolog of the protein as defined in (i) having an amino acid sequence with at least 80% similarity to one of said protein after optimal alignment; and

(iii) a peptide fragment of the protein as defined in (i) or (ii) having a T-cell epitope or chemical analog thereof; and one or more pharmaceutically acceptable adjuvants, carriers, excipients and/or diluents.

According to the vaccine of the present invention, the *Mycobacterium* species is selected from *M. tuberculosis*, *M. bovis*, *M. bovis* BCG, *M. africanum*, *M. canetti*, *M. caprae*, *M. microti*, *M. pinnipedii*, *M. avium*, *M. avium paratuberculosis*, *M. avium silvaticum*, *M. avium* "hominissuis", *M. colombiense*, *M. asiaticum*, *M. gordonae*, *M. gastris*, *M. kansasii*, *M. hiberniae*, *M. nonchromogenicum*, *M. terrae*, *M. triviale*, *M. ulcerans*, *M. pseudoshottsii*, *M. shottsii*, *M. triplex*, *M. genavense*, *M. florentinum*, *M. lentiflavum*, *M. palustre*, *M. kubicae*, *M. parascrofulaceum*, *M. heidelbergense*, *M. interjectum*, *M. simiae*, *M. branderi*, *M. cookii*, *M. celatum*, *M. bohemicum*, *M. haemophilum*, *M. malmoense*, *M. szulgai*, *M. leprae*, *M. lepraemurium*, *M. lepromatosis*, *M. africanum*, *M. botniense*, *M. chimaera*, *M. conspicuum*, *M. doricum*, *M. farcinogenes*, *M. heckeshornense*, *M. intracellulare*, *M. lacus*, *M. marinum*, *M. monacense*, *M. montefiorensis*, *M. murale*, *M. nebraskense*, *M. saskatchewanense*, *M. scrofulaceum*, *M. shimoidei*, *M. tusciae*, *M. xenopi*, *M. intermedium*, *M. abscessus*, *M. chelonae*, *M. bolletii*, *M. fortuitum*, *M. fortuitum* subsp. *acetamidolyticum*, *M. boenickei*, *M. peregrinum*, *M. porcinum*, *M. senegalense*, *M. septicum*, *M. neworleansense*, *M. houstonense*, *M. mucogenicum*, *M. mageritense*, *M. brisbanense*, *M. cosmeticum*, *M. parafortuitum*, *M. austroafricanum*, *M. diernhoferi*, *M. hodleri*, *M. neoaurum*, *M. frederiksbergense*, *M. aurum*, *M. vaccae*, *M. chitae*, *M. fallax*, *M. confluentis*, *M. flavescens*, *M. madagascariense*, *M. phlei*, *M. smegmatis*, *M. goodii*, *M. wolinskyi*, *M. thermoresistibile*, *M. gadium*, *M. komossense*, *M. obuense*, *M. sphagni*, *M. agri*, *M. aichiense*, *M. alvei*, *M. arupense*, *M.*

brumae, *M. canariense*, *M. chubuense*, *M. conceptionense*, *M. duvalii*, *M. elephantis*, *M. gilvum*, *M. hassiacum*, *M. hol-saticum*, *M. immunogenum*, *M. massiliense*, *M. moriokaense*, *M. psychrotolerans*, *M. pyrenivorans*, *M. vanbaalenii*, *M. pulveris*, *M. arosiense*, *M. aubagnense*, *M. caprae*, *M. chlorophenicum*, *M. fluoroanthenvivorans*, *M. kumamotoense*, *M. novocastrense*, *M. parmense*, *M. phocaicum*, *M. poriferae*, *M. rhodesiae*, *M. seoulense* and *M. tokaiense*. Preferably, the *Mycobacterium* species is *Mycobacterium tuberculosis*.

Vaccine of the present invention can be used in human or non-human animal subject. The peptide as defined in (iii) can comprise or consist of an amino acid sequence selected from TAWITAWPGLMV (SEQ ID NO:24), AVIVRSELLTQYL (SEQ ID NO:22), GSVRQLPSVLKPLITLRLTLG (SEQ ID NO:71), RPVRRVLLFWPSSGPAP (SEQ ID NO:70), GEIIFISGRLNGaa (SEQ ID NO:13), ELMA-RAAVLGSAAH (SEQ ID NO:21), LAWITAWPGLMV (SEQ ID NO:85), GEIIFISGRLNG (SEQ ID NO:86), SALLRRL-STCPPES (SEQ ID NO:87). Therefore, the present invention concerns vaccine as defined above for use in the prevention of infection by *Mycobacterium* species.

It is further object of the present invention, at least one agent selected from the list consisting of:

(i) a protein selected from Rv0023, Rv0182c, Rv0290, Rv0601c, Rv0647c, Rv0724A, Rv0890c, Rv1251c, Rv1398c, Rv1478, Rv1497, Rv1575, Rv1578c, Rv1899c, Rv2137c, Rv2333c, Rv2548, Rv2557, Rv2816c, Rv2990, Rv3094c, Rv3107c, Rv3188, Rv3239c, Rv3296, Rv3425, Rv3446c, Rv3479, Rv3482c, Rv3780, derived from a *Mycobacterium* species or related organism and comprising at least one T-cell epitope;

(ii) an homolog of the protein as defined in (i) having an amino acid sequence with at least 80% similarity to one of said protein after optimal alignment; and

(iii) a peptide fragment of the protein as defined in (i) or (ii) having a T-cell epitope or a chemical analog thereof; for use in the treatment or prevention of infection by *Mycobacterium* species.

The present invention further concerns a method for in vitro assessing the capacity for a subject to mount a cell-mediated immune response, said method comprising contacting a sample comprising T-lymphocytes sensitized to *Mycobacterium* species or antigens or proteins comprising T-lymphocyte epitopes derived therefrom with at least one agent selected from:

(i) a protein selected from Rv0023, Rv0182c, Rv0290, Rv0601c, Rv0647c, Rv0724A, Rv0890c, Rv1251c, Rv1398c, Rv1478, Rv1497, Rv1575, Rv1578c, Rv1899c, Rv2137c, Rv2333c, Rv2548, Rv2557, Rv2816c, Rv2990, Rv3094c, Rv3107c, Rv3188, Rv3239c, Rv3296, Rv3425, Rv3446c, Rv3479, Rv3482c, Rv3780, derived from a *Mycobacterium* species or related organism and comprising at least one T-cell epitope;

(ii) an homolog of the protein as defined in (i) having an amino acid sequence with at least 80% similarity to one of said protein after optimal alignment; and

(iii) a peptide fragment of the protein as defined in (i) or (ii) having a T-cell epitope or a chemical analog thereof; for a time and under conditions sufficient to stimulate the lymphocytes to produce an effector molecule, wherein the presence or level of the effector molecule is indicative of the subject's capacity to mount a cell-mediated immune response.

The present invention now will be described by an illustrative, but not limitative way, according to preferred embodiments thereof, with particular reference to enclosed drawings wherein:

FIG. 1. Analysis of IFN-gamma production as PPD response comparing four subject populations: a. first test pulmonary TB patients; b. healthy contacts, TB exposed, PPD positive; c. healthy controls, professionally TB exposed, Quantiferon positive; d. negative controls, Quantiferon negative, BCG vaccinated.

FIG. 2. Analysis of IFN-gamma production as TAWITAW-PGLMV (SEQ ID NO 24) peptide response from 4 tested subject groups.

FIG. 3. Analysis of IFN-gamma production as AVIVRSELLTQYL (SEQ ID NO 22) peptide response from 4 tested subject groups.

FIG. 4. Analysis of IFN-gamma production as GSVRQLPSVLKPPLTLRRTLTLG (SEQ ID NO 71) peptide response from 4 tested subject groups.

FIG. 5. Analysis of IFN-gamma production as RPVRRV-LLFWPSSGPAP (SEQ ID NO 70) peptide response from 4 tested subject groups.

FIG. 6. Analysis of IFN-gamma production as GEIIFIS-GRLNGaa (SEQ ID NO 13) peptide response of tested samples.

FIG. 7. Analysis of IFN-gamma production as ELMA-RAAVLGSAH (SEQ ID NO 21) peptide response of tested samples.

FIG. 8. Analysis of IFN-gamma production as ESAT6 (QQWNFAGIEAAASAIQGNVTSIHSL—SEQ ID NO:84) response of tested samples.

FIG. 9. Frequency of positive tests using only SEQ ID NO:24, 21, 71, 70, 13, 22 six peptides.

FIG. 10. Sensitivity increase of Quantiferon TB-plus commercial used test after SEQ ID NO: 24, 21, 70, 71, 13, 22 peptide addition thereto.

FIG. 11. Combination use of 1-6 peptides, which allow in panel a 76.3%, 62% and 66.7%, compared to 55%, 33% and 45.5% sensitivity obtained using RD1 (ESAT6) multiepitope peptide, for TB+ subjects, PPD+ exposed contacts and HCWs, respectively, to be obtained.

EXAMPLE 1

Identification of *M. Tuberculosis* Expressed Proteins in Human Infected Macrophages from Both Vitro and Ex Vivo Analyzed Biological Samples

Materials and Methods

ELISPOT Immunodiagnostic Test

The global procedure for test carrying out demands for: 96 well plates (MAIPS45, Millipore, Sunnyvale, Calif., USA); primary antibody (IFN-gamma coating monoclonal, M-700A, Pierce-Endogen Inc, Rockford, USA); biotinylated antibody (M-701B, Pierce-Endogen Inc); streptavidin-HRP (Pierce-Endogen); substrate (AEC Staining kit, Sigma); stimulus (peptides, PHA and other antigens) at ready to use concentration.

ELISPOT procedure is carried out according to the following step:

Coating: treatment of a 96 well plate using 5 µg/mL sterile phosphate buffer (PBS) solution of primary antibody delivered at 100 µL/well; coat the plate and incubate at 4° C. for 20 hours; wash the plate 4 times using 200 µL/well of sterile PBS, at final washing eliminate excess liquid by tapping plate on adsorbent paper.

Blocking: addition of 200 µL/well of “blocking solution” [sterile PBS containing 10% fetal calf serum (FCS)], in order to prevent not specific protein binding; plate incubation for 2 hours at room temp.; “blocking solution” suction.

Cell Preparation and Incubation

1. isolation of mononucleated cells (PBMC) from venous blood (7 ml with EDTA) by density gradient centrifugation (Ficoll-Hypaque, Pharmacia; Uppsala; Sweden), using a quick method based on the use of filtering tubes for leucocytes separation (LeucoSep™, ARNIKA, Milan). After two washings with PBS (phosphate buffered saline) 1x, the pellet is resuspended in complete medium (RPMI 1640 containing HEPES 25 mM, 10% v/v FCS, 2 mM L-Glutamine, 10 U/mL penicillin/streptomycin) in order to have 2x10⁵ cells in 100 µL.

2. add 100 µL/well of cellular suspension together with 100 µL of different stimula;

3. incubate the plate for 40 hours at 37° C. in incubator at 5% CO₂;

4. remove the cells;

5. wash the plate 4 times with 200 µL/well of PBS and 4 times with 200 µL/well of “Wash buffer” [PBS/0.05% Tween 20 (Sigma)];

6. at the final washing eliminate the excess liquid by tapping the plate on adsorbent paper.

Incubation with Biotinylated Antibody

100 µL/well of biotinylated antibody diluted in PBS/4% bovine serum albumin (fraction V, Sigma) at concentration of 1 µg/ml have been delivered. Then the plate has been incubated for 100 minutes at 37° C. in incubator at 5% CO₂; the plate has been washed 4 times with “Wash buffer”. At final washing excess liquid has been eliminated by tapping plate on adsorbent paper.

Detection

For the detection 100 µL/well of “Streptavidin-HRP” 1:1000 diluted in “Wash buffer”. The plate has been incubated for 30 minutes at room temp. in the dark, then washed 4 times with “Wash buffer”. At final washing excess liquid has been eliminated by tapping plate on adsorbent paper. 100 µL/well of substrate have been delivered. In parallel as a control for enzyme-substrate reaction occurrence 100 µL of as prepared substrate with 100 µL of “diluted Streptavidin-HRP” has been incubated for few minutes. If the reaction is successful the substrate turns out from light brown to pink colour.

Finally the plate has been incubated for 10-20 minutes at room temp. in the dark. The substrate has been discarded, the plate washed with water eliminating excess and air dried for 20 hours.

ELISA tests for the identification of human and animal IFN-gamma in whole blood samples stimulated with selected peptides and proteins (CMI test protocol).

Results

The authors have identified the group of *M. tuberculosis* expressed proteins in human infected macrophages from both in vitro and ex vivo analyzed biological samples. *M. tuberculosis* gene expressions in three different growth environments have been compared: synthetic medium (Sauton's), monocyte-derived-human macrophage (MDM) infected in culture with *M. tuberculosis*, alveolar macrophage (AM) from bronchoalveolar lavage (BAL) samples of pulmonary TB affected patients before the antibiotic therapy.

From thus obtained 9 groups of genes, first 100 proteins, according to a combination criteria (immunogenicity, tubercular Complex specificity, etc), have been selected. From these 100 proteins, again a 30 protein group has been selected, for which a positive response in immunological tests on TB patient whole blood had been obtained.

TABLE 1

Rv	In vivo modulation	Amino acid sequence ID (UNIPROT)	Already tested peptides
Rv0023	B, E	P67704	EMWDIRNRGVIPAGALPRVR (SEQ ID NO: 1)
Rv0182c	E	O07426	AKFRSVRVVVITGSVTAAPVRVSETLRRLI (SEQ ID NO: 2), ESVRLAFVAALQH (SEQ ID NO: 3)
Rv0290	D	O86362	GLLITIRSPRSGIA (SEQ ID NO: 4), AQLLWQLPLLSIG (SEQ ID NO: 5)
Rv0601c	C, E	O07777	ADLVRELVTILPIVLVIAAVALLSR (SEQ ID NO: 6) AAYLLSRAALRPVDRIRAAA (SEQ ID NO: 7) TTLNMLTRLQRALAHEQQF (SEQ ID NO: 8) DLFVSDIPDHLRRLTAVLDN (SEQ ID NO: 9) SGLGLAIVAAALTTTHGG (SEQ ID NO: 10)
Rv0647c	E	P96936	GRLPRKGPWQQKVIKELPQ (SEQ ID NO: 11), GKIVVLMGAVGTMKQPETQAA (SEQ ID NO: 12)
Rv0724A	B	Q79FX1	GEIIFISGRNLGaa (SEQ ID NO: 13)
Rv0890c	C, E	Q10550	ARVRSMSPLEIAD (SEQ ID NO: 14), EQILFRRLAPFVGGF (SEQ ID NO: 15), AALVRALTACGCSS (SEQ ID NO: 16), DKWTLQCILYWRGVGTCISGD (SEQ ID NO: 17), TKVLGLYTQAQVLAFCG (SEQ ID NO: 18), DQVTMHQVLMALALAGG (SEQ ID NO: 19), EGVRLLGAAAALRQQTRQVRFK (SEQ ID NO: 20)
Rv1251c	C, E	O50466	ELMARAVALGSAAH (SEQ ID NO: 21), AVIVRSELLTQYL (SEQ ID NO: 22)
Rv1398c	D	P64835	GTLRHLDPPVRRSGGREQHL (SEQ ID NO: 23)
Rv1478	E	O53169	TAWITAVVPGLMV (SEQ ID NO: 24)
Rv1497	C, E	P71778	APMVFSATKGMTA (SEQ ID NO: 25), TCAMRRLAHRFSGG (SEQ ID NO: 26)
Rv1575	C, E	O06615	SVVRRKQTLLSAQ (SEQ ID NO: 27)
Rv1578c	E	O06612	GVVHRNPAVTVAE (SEQ ID NO: 28)
Rv1899c	D	O07733	PGVVATHAVRTLGGTGSRAIGL (SEQ ID NO: 29), PQWRRARVRLCGRWRRSNTTRGAS (SEQ ID NO: 30), ARLMVGAVRRHRPGSLQR (SEQ ID NO: 31)
Rv2137c	D	O06238	aaMRNMKSTSHE (SEQ ID NO: 32)
Rv2333c	E	P71879	QTIVMLWTAAVGCA (SEQ ID NO: 33), LCMLMLGLLMLIFSEHRSS (SEQ ID NO: 34), SALVLVGLGLCGSGVALCLT (SEQ ID NO: 35)
Rv2548	A, E	P95005	SELVRFELLAGVRESE (SEQ ID NO: 36), VDYLIAATAIVVDA (SEQ ID NO: 37)
Rv2557	A, E	P65003	QGIEYYKSSVLPQIE (SEQ ID NO: 38), EGWIVYARSTTIQAQS (SEQ ID NO: 39), TRRMYSNYGF (SEQ ID NO: 40)
Rv2816c	A	P71637	FGYRVQESAFEAMLTGKQLAKLV (SEQ ID NO: 41), DNIRIYKIRGVAAVTFYGRGRLVSAE (SEQ ID NO: 42)
Rv2990	E	O53239	RSYILRAGISSLFRYIEGVHGER (SEQ ID NO: 43), SAMRPQDRLLVGNWVDDSL (SEQ ID NO: 44), LYLVGLEPYVQFE (SEQ ID NO: 45), AGFRILEARRFPI (SEQ ID NO: 46), IRYRARYVNGQLNMCLARI (SEQ ID NO: 47)
Rv3094c	E	O05773	ALLVAYLPARSREEMF (SEQ ID NO: 48), NRLRLAATHAVRT (SEQ ID NO: 49), APLQRRFRDAFTATAHFQVNE (SEQ ID NO: 50), SRELPGRVLLDQPADVSM (SEQ ID NO: 51)
Rv3107c	A, E	O05784	EPVVTVDVTAMSAVLEID (SEQ ID NO: 52)

TABLE 1-continued

Rv	In vivo modulation	Amino acid sequence ID (UNIPROT)	Already tested peptides
Rv3188	C, E	O53334	AVIQVSDRAVRGWRTGDIRPERY (SEQ ID NO: 53)
Rv3239c	C, E	O05884	PDLRGALLLAVTLGLVT (SEQ ID NO: 54), PDWGWLSVATVGSFLA (SEQ ID NO: 55), GAVLGVAVMVILIGKPEHGTA (SEQ ID NO: 56), AAICFIAVAVAAAVL (SEQ ID NO: 57), TKLVRLTKAQFDEIA (SEQ ID NO: 58), ADLVLAGPAASREH (SEQ ID NO: 59), YAYEYFIRHNPPLSDYA (SEQ ID NO: 60), FPVRLVRRRRTLLEA (SEQ ID NO: 61)
Rv3296	A, E	P96901	EVLRLRRRSLAALRA (SEQ ID NO: 62), RVILHSPYGLRVHGPLAL (SEQ ID NO: 63)
Rv3425	E	Q50703	AAWINGLANAYNDT (SEQ ID NO: 64), DQYRARNVAVMNAYVSWTRALSDDLPR (SEQ ID NO: 65), SLLADAVERYLQWLKSSSQLKHA (SEQ ID NO: 66)
Rv3446c	C, E	O06263	GPVVVHPSWSAA (SEQ ID NO: 67), ITAVVLIDVPSTVAGA (SEQ ID NO: 68), AAVVRHGATTLQRP (SEQ ID NO: 69)
Rv3479	C, E	O06342	RPVRRVLLFVVPSSGPAP (SEQ ID NO: 70), GSVRQLPSVLKPLITLRTLTLG (SEQ ID NO: 71), SALLRRLSTCPPES (SEQ ID N: 87)
Rv3482c	E	O06345	GAVLRLVVRFAEPLPPSP (SEQ ID NO: 72), AGYLLTYTIANNKKEFAEL (SEQ ID NO: 73)
Rv3780	D	P65091	aaVRKRMVIGLSTGSDDD (SEQ ID NO: 74)
Cons FS			ALLLRDVLQWKSAEVADAIG (SEQ ID NO: 75) NSLLQRARSQLOTVRPSAADRLSAA (SEQ ID NO: 76)
Cons PE_PGRS			MSWVMVSPPELVVAAAADLAG (SEQ ID NO: 77) AAFYAQFVQALTSGGAY (SEQ ID NO: 78)
Cons REG			ALLVRMPTSLPAVA (SEQ ID NO: 79)
Cons CW			SRLRTHVRPDAPLPLALRVDGLRSRW (SEQ ID NO: 80) AAVLTMLGVAGYGW (SEQ ID NO: 81) GLFMIFLDALIVNVALPDIQR (SEQ ID NO: 82) SWVVASYSGLMAVFIMSAGTLADLL (SEQ ID NO: 83)

Legend of modulation:

- A: up-regulated in AM vs MDM;
B: always expressed in AM and MDM;
C: up-regulated in MDM vs AM;
D: up-regulated in Sauton vs MDM and/or AM;
E: up-regulated in MDM and/or AM vs Sauton's

After an ulterior selection initially 43 peptides from 4 groups of subjects: Pulmonary TB before antibiotic therapy (n=13), recently exposed healthy contacts (TB patient relations) PPD+(n=8); long TB patient exposed healthy contacts (professional exposure of hospital workers) PPD+(n=5); BCG vaccinated negative controls, PPD- (n=4), have been designed, synthesized and tested.

Then 6 most sensible and specific peptides have been selected (see table 2) and the study has been repeated using an extended subject sample (see tables 3-4 and FIGS. 1-7).

Table 2 reports MTB selected genes, peptides selected for T CD4+ cell assay and corresponding identification number thereof, respectively.

TABLE 2

N°	Peptide	Gene	In vivo modulation
SEQ ID NO: 24	TAWITAVVPGLMV	Consensus VIR(Rv1478)	Induced in AM and MDM vs Sauton's

TABLE 2-continued

N°	Peptide	Gene	In vivo modulation
SEQ ID AVIVRSELLTQYL NO: 22		Rv1251c	Induced in MDM vs Sauton's
SEQ ID GSVRQLPSVLKPPILTLRITLTL NO: 71 SG		Rv3479	Induced in MDM vs Sauton's
SEQ ID RPVRRVLLFVVPSSGPAP NO: 70		Rv3479	Induced in MDM vs Sauton's
SEQ ID GEIIFISGRNLGaa NO: 13		Rv0724A	Expressed in AM and MDM
SEQ ID ELMARAAVLGSAH NO: 21		Rv1251c	Induced in MDM vs Sauton's

TABLE 3

	Micro-biologically checked TB patients (before therapy start)	Recently MTB exposed subjects	Sanitary workers	BCG-vaccinated controls
Examined subject number	58	63	21	15
Anergic subjects (mitogene low response or no response to all stimula)	3/58	2/63	0/21	0/15
Quantiferon TB-Gold in-Tube PPD	41/55	25/61	11/21	3/15
ESAT-6 control peptide	55/55	61/61	21/21	15/15
Peptide #1 (Seq ID N°: 24)	28/55	17/61	7/21	0/15
Peptide #2 (Seq ID N°: 22)	12/55	14/61	6/21	0/15
Peptide #3 (Seq ID N°: 71)	11/55	8/61	1/21	0/15
Peptide #4 (Seq ID N°: 70)	27/55	20/61	7/21	0/15
Peptide #5 (Seq ID N°: 13)	10/55	13/61	6/21	0/15
Peptide #6 (Seq ID N°: 21)	18/55	11/61	5/21	0/15
	14/55	6/61	3/21	0/15

TABLE 4

	Micro-biologically checked TB patients (before therapy start)	Recently MTB exposed subjects	Sanitary workers	BCG-vaccinated controls
Examined subject number	58	63	21	15
Anergic subjects (mitogene low response or no response to all stimula)	3/58	2/63	0/21	0/15
Quantiferon TB-Gold in-Tube Panel 1-6 peptides	41/55	25/61	11/21	3/15
QFT Gold + panel 1-6 peptides	40/55	30/61	11/21	0/15
	49/55	34/61	13/21	3/15

TABLE 5

	Micro-biologically checked TB patients (before therapy start)	Recently MTB exposed subjects	Sanitary workers	BCG-vaccinated controls
Examined subject number	38	32	10	10
Anergic 1-6 peptides (in pool)	3	1	0	0
QFT Gold	22/35	10/31	4/10	1/10
QFT Gold + 1-6 peptides in same well	25/35	11/31	4/10	2/10
	29/35	13/31	5/10	2/10

Results obtained with said six peptides and peptide belonging to ESAT6 protein, i.e. a highly immunogenic protein occurring in both above mentioned commercial kits, have been compared.

MTB genes observed as induced, both in course of human macrophage infection and/or in alveolar macrophage samples from active pulmonary TB patients, are indicated in the following list:

Genes always expressed during intracellular replication in MDM and AM: Rv0724A.

Genes induced in AM and/or MDM vs Sauton's medium culture: Rv1251c, Rv1478 and Rv3479.

Two groups of MTB genes share probable role in the survival inside human host cell (both primary macrophages from healthy in vitro infected donors and TB patient alveolar macrophages) resulting in design thereof as MTB intracellular survival bio-markers, whereas MTB virulence definition is just based on the pathogen ability to invade, survive and replicate within the host cell.

Moreover, the authors of the present invention have designed peptides of some gene groups belonging to same metabolic category, in order to find "consensus" protein sequences for said categories. The research is based on the assumption that functional domains of similar function exploiting proteins occurring in various bacterial species, are conserved. In order to find these conserved motifs the sequence multiple alignment (PSSM), using PSI-BLAST (Position Specific Iterated Basic Local Alignment Search Tool, Altschul et al., 1997 *Nucl. Ac. Res.* 25:3389; URL: world wide web.ncbi.nlm.nih.gov/BLAST) is generated. After the sequences with higher similarity grades with the inserted sequence has been detected, it is possible to select the proteins suitable to contribute to the generation of the profile

used for the successive data bank search; in this way the number of the sequences contributing to the generation of the profile is different for the different sequence positions.

A multi-alignment allows structurally and functionally important, because extremely conserved, residues to be detected and said residues as a whole will constitute the "consensus" sequence or sequences for each MTB protein functional group.

Therefore proteins (induced or repressed in human macrophage) of metabolic functional groups (for example regulatory proteins, lipid metabolism involved proteins, etc.) detected as "modulated" by *M. tuberculosis* during infection course, have been analyzed for search of conserved sequences. Using PSI-BLAST sequence various multiple alignments, from which we have reached the best "consensus" sequences for the peptide synthesis, have been obtained.

The peptides derived from selected proteins have been synthesized and used for the detection and quantification of MTB specific T CD4+ lymphocytes using detection system for IFN- γ producing cells both with ELISPOT technique and with TB diagnosis high sensitivity ELISA assay, Quantiferon TB-Plus and Quantiferon CMI. This technique allows the frequency of T cells producing a determined cytokine (for example, IFN- γ) as a response to a specific antigenic stimulus suggesting that the immune system of treated subjects has been able to evoke an immune response towards said peptides when infectious agent (MTB) encoding for the same occurs, to be quantified. The second technique allows total IFN- γ production resulting from specific T lymphocytes as a response to selected antigens, to be quantified.

Although this test does not represent the evidence of ability thereof to induce protection from MTB infection, the occurred detection of the presence of lymphocytes recognizing these peptides specifically and differently in MTB infected subjects or with active tuberculosis, is an index of their immunogenicity, indispensable minimal characteristic in order a vaccine and a diagnostic test to be proved effective. Further these peptides, alone or in addition to other mycobacterial antigens, allow a sensitive and specific test for TB diagnosis to be provided and the sensitivity of commercial test, i.e. current reference gold standard for the tubercular diagnosis, to be enhanced (FIG. 10). When panel evaluated and directly tested within same well, 6 selected peptides allow Quantiferon TB gold in-Tube response from 75% to 89% (+14%) and from 71% to 83% (+13%), respectively, for subjects with active TB, to be enhanced, without decreasing the assay specificity.

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Pro Arg Val Arg
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Arg Ala Ala Ala
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<210> SEQ ID NO 8
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 <212> TYPE: PRT
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Glu Gln Gln Phe
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<212> TYPE: PRT

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<210> SEQ ID NO 11

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Leu Pro Gln

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<211> LENGTH: 20

<212> TYPE: PRT

<213> ORGANISM: Mycobacterium tuberculosis

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<210> SEQ ID NO 13

<211> LENGTH: 14

<212> TYPE: PRT

<213> ORGANISM: Mycobacterium tuberculosis

<400> SEQUENCE: 13

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<210> SEQ ID NO 14

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<212> TYPE: PRT

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<212> TYPE: PRT

<213> ORGANISM: Mycobacterium tuberculosis

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Cys Ile Ser Gly Asp
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<211> LENGTH: 17

<212> TYPE: PRT

<213> ORGANISM: Mycobacterium tuberculosis

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<210> SEQ ID NO 19

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<212> TYPE: PRT

<213> ORGANISM: Mycobacterium tuberculosis

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<213> ORGANISM: Mycobacterium tuberculosis

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<400> SEQUENCE: 22

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Glu Gln His Leu
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<213> ORGANISM: Mycobacterium tuberculosis

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<210> SEQ ID NO 25
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<213> ORGANISM: Mycobacterium tuberculosis

<400> SEQUENCE: 25

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<400> SEQUENCE: 26

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<210> SEQ ID NO 27
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<212> TYPE: PRT
<213> ORGANISM: Mycobacterium tuberculosis

<400> SEQUENCE: 27

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Ser Val Val Arg Arg Lys Gln Thr Leu Leu Ser Ala Gln
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<210> SEQ ID NO 28
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 <212> TYPE: PRT
 <213> ORGANISM: Mycobacterium tuberculosis
 <400> SEQUENCE: 28

Gly Val Val His Arg Asn Pro Ala Val Thr Val Ala Glu
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<210> SEQ ID NO 29
 <211> LENGTH: 22
 <212> TYPE: PRT
 <213> ORGANISM: Mycobacterium tuberculosis
 <400> SEQUENCE: 29

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Ser Arg Ala Ile Gly Leu
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Arg Ser Asn Thr Thr Arg Gly Ala Ser
 20 25

<210> SEQ ID NO 31
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 <212> TYPE: PRT
 <213> ORGANISM: Mycobacterium tuberculosis
 <400> SEQUENCE: 31

Ala Arg Leu Met Val Gly Ala Val Arg Arg His Arg Pro Gly Ser Leu
 1 5 10 15

Gln Arg

<210> SEQ ID NO 32
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 <212> TYPE: PRT
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 <212> TYPE: PRT
 <213> ORGANISM: Mycobacterium tuberculosis
 <400> SEQUENCE: 33

Gln Thr Ile Val Met Leu Trp Thr Ala Ala Val Gly Cys Ala
 1 5 10

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Leu Cys Met Leu Met Leu Gly Leu Leu Met Leu Ile Phe Ser Glu His
 1 5 10 15

Arg Ser Ser

<210> SEQ ID NO 35
 <211> LENGTH: 20
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<400> SEQUENCE: 35

Ser Ala Leu Val Leu Val Gly Leu Gly Leu Cys Gly Ser Gly Val Ala
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Leu Cys Leu Thr
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<210> SEQ ID NO 36
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<400> SEQUENCE: 36

Ser Glu Leu Val Arg Phe Glu Leu Leu Ala Gly Val Arg Glu Ser Glu
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<210> SEQ ID NO 37
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<400> SEQUENCE: 37

Val Asp Tyr Leu Ile Ala Ala Thr Ala Ile Val Val Asp Ala
 1 5 10

<210> SEQ ID NO 38
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<400> SEQUENCE: 38

Gln Gly Ile Glu Tyr Tyr Lys Ser Ser Val Leu Pro Gln Ile Glu
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<210> SEQ ID NO 39
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<400> SEQUENCE: 39

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 1 5 10 15

<210> SEQ ID NO 40
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<400> SEQUENCE: 40

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1 5 10

<210> SEQ ID NO 41
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 <212> TYPE: PRT
 <213> ORGANISM: Mycobacterium tuberculosis

<400> SEQUENCE: 41

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Gly Gln Leu Ala Lys Leu Val
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<210> SEQ ID NO 42
 <211> LENGTH: 26
 <212> TYPE: PRT
 <213> ORGANISM: Mycobacterium tuberculosis

<400> SEQUENCE: 42

Asp Asn Ile Arg Ile Tyr Lys Ile Arg Gly Val Ala Ala Val Thr Phe
 1 5 10 15

Tyr Gly Arg Gly Arg Leu Val Ser Ala Glu
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<210> SEQ ID NO 43
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<400> SEQUENCE: 43

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Glu Gly Val His Gly Glu Arg
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<210> SEQ ID NO 44
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<400> SEQUENCE: 44

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Asp Ser Leu Leu
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<210> SEQ ID NO 46
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<400> SEQUENCE: 46

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<210> SEQ ID NO 47
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 <212> TYPE: PRT
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Ala Arg Ile

<210> SEQ ID NO 48
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<400> SEQUENCE: 48

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Ile Asp

<210> SEQ ID NO 53
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<212> TYPE: PRT
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Thr

<210> SEQ ID NO 55
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<210> SEQ ID NO 56
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Glu His Gly Thr Ala
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<210> SEQ ID NO 59
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Glu Ala

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Ala Leu

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Trp Thr Arg Ser Ala Leu Ser Asp Leu Pro Arg
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<210> SEQ ID NO 66
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<212> TYPE: PRT
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Lys Ser Ser Ser Gln Leu Lys His Ala
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<400> SEQUENCE: 67

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<210> SEQ ID NO 68
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<400> SEQUENCE: 68

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<210> SEQ ID NO 69
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<400> SEQUENCE: 69

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Ala Pro

<210> SEQ ID NO 71
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Leu Arg Thr Leu Thr Leu Ser Gly
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<210> SEQ ID NO 72
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Ser Pro

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Ala Glu Leu

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Asp Ala Ile Gly
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Ser Ala Ala Asp Arg Leu Ser Ala Ala
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Asp Leu Ala Gly
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Tyr

<210> SEQ ID NO 79

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<212> TYPE: PRT

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<210> SEQ ID NO 80

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<213> ORGANISM: Mycobacterium tuberculosis

<400> SEQUENCE: 80

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Ala Leu Arg Val Asp Gly Leu Arg Ser Arg Trp
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<210> SEQ ID NO 81

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<212> TYPE: PRT

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<400> SEQUENCE: 81

Ala Ala Val Leu Thr Met Leu Gly Val Ala Gly Tyr Gly Trp
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<210> SEQ ID NO 82

<211> LENGTH: 21

<212> TYPE: PRT

<213> ORGANISM: Mycobacterium tuberculosis

<400> SEQUENCE: 82

Gly Leu Phe Met Ile Phe Leu Asp Ala Leu Ile Val Asn Val Ala Leu
 1 5 10 15

Pro Asp Ile Gln Arg
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<210> SEQ ID NO 83

<211> LENGTH: 25

<212> TYPE: PRT

<213> ORGANISM: Mycobacterium tuberculosis

<400> SEQUENCE: 83

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Ser Ala Gly Thr Leu Ala Asp Leu Leu
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<210> SEQ ID NO 84

<211> LENGTH: 25

<212> TYPE: PRT

<213> ORGANISM: Mycobacterium tuberculosis

<400> SEQUENCE: 84

-continued

Gln Gln Trp Asn Phe Ala Gly Ile Glu Ala Ala Ala Ser Ala Ile Gln
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Gly Asn Val Thr Ser Ile His Ser Leu
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<210> SEQ ID NO 85
<211> LENGTH: 13
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<400> SEQUENCE: 85

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<210> SEQ ID NO 86
<211> LENGTH: 12
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<213> ORGANISM: Mycobacterium tuberculosis

<400> SEQUENCE: 86

Gly Glu Ile Ile Phe Ile Ser Gly Arg Leu Asn Gly
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<210> SEQ ID NO 87
<211> LENGTH: 14
<212> TYPE: PRT
<213> ORGANISM: Mycobacterium tuberculosis

<400> SEQUENCE: 87

Ser Ala Leu Leu Arg Arg Leu Ser Thr Cys Pro Pro Glu Ser
1 5 10

The invention claimed is:

1. A method for detecting a *Mycobacterium tuberculosis* infection in a test subject, comprising:

(A) incubating in vitro (i) a blood sample or a lavage sample that comprises lymphocytes from the test subject, with (ii) ESAT6 protein and CFP10 protein, and (iii) at least one of:

(a) at least 6 peptides that each comprise at least one *Mycobacterium tuberculosis* T-cell epitope, said peptides being selected from TAWITAWPGLMV (SEQ ID NO:24), ELMARA AVLGSAH (SEQ ID NO:21), RPVRRVLLFWPSSGPAP (SEQ ID NO:70), GSVRQLPSVLKPPLITLRTLTLG (SEQ ID NO:71), GEIIFISGRLNGaa (SEQ ID NO: 13), AVIVRSELLTQYL (SEQ ID NO:22), LAWITAWPGLMV (SEQ ID NO:85), GEIIFISGRLNG (SEQ ID NO:86) and SALLRRLSTCPPES (SEQ ID NO:87),

(b) at least 6 peptides that each comprise at least one *Mycobacterium tuberculosis* T-cell epitope, said peptides being selected from ELMARA AVLGSAH (SEQ ID NO:21), RPVRRVLLFWPSSGPAP (SEQ ID NO:70), GSVRQLPSVLKPPLITLRTLTLG (SEQ ID NO:71), AVIVRSELLTQYL (SEQ ID NO:22), TAWITAWPGLMV (SEQ ID NO:24) and GEIIFISGRLNGaa (SEQ ID NO:13), and

(c) at least 6 peptides that each comprise at least one *Mycobacterium tuberculosis* T-cell epitope, said peptides being selected from ELMARA AVLGSAH (SEQ ID NO:21), RPVRRVLLFWPSSGPAP (SEQ ID NO:70), GSVRQLPSVLKPPLITLRTLTLG (SEQ ID NO:71), AVIVRSELLTQYL (SEQ ID

NO:22), LAWITAWPGLMV (SEQ ID NO:85) and GEIIFISGRLNG (SEQ ID NO:86),

wherein said step of incubating is for a time and under conditions sufficient for:

(1) recognition, by one or more of a first plurality of T cell receptors (TCR) of T cells that are present in said lymphocytes, of one or more of said at least 6 peptides of (a), (b), or (c) to stimulate the lymphocytes to produce an effector molecule, and

(2) recognition, by one or more of a second plurality of T cell receptors (TCR) of T cells that are present in said lymphocytes, of one or more of said ESAT6 and CFP10 proteins to stimulate the lymphocytes to produce the effector molecule; and

(B) detecting a test level of the effector molecule that is produced by lymphocytes stimulated according to step (A), wherein the detected test level of the effector molecule is greater than a detectable effector molecule level that can be produced by said lymphocytes incubated only with said ESAT6 and CFP10 proteins,

wherein if the test level of the effector molecule that is detected is higher than a control level of the effector molecule that is detected following incubation of control lymphocytes from a healthy control subject, then the test level indicates that the test subject is infected with, or has been previously exposed to, *Mycobacterium tuberculosis*, and wherein the effector molecule is selected from (i) a cytokine or an interleukin the test level of which is indicative of the test subject's capacity to mount a cell-mediated immune response, or (b) IFN- γ or TNF- α .

2. The method of claim 1 in which incubating further comprises incubating with TB7.7 protein for a time and under

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conditions sufficient for recognition, by one or more of said second plurality of T cell receptors (TCR) of T cells that are present in said lymphocytes, of said TB7.7 protein to stimulate the lymphocytes to produce the effector molecule, further wherein the detected test level of the effector molecule is greater than a detectable effector molecule level that can be produced by lymphocytes incubated only with said ESAT6, CFP10 and TB7.7 proteins. 5

3. The method of claim 1 wherein the effector molecule is selected from interferon- γ , TNF- α , and an interleukin. 10

4. The method of claim 1 wherein the effector molecule is interferon- γ .

* * * * *

专利名称(译)	使用来自结核分枝杆菌或相应核酸的氨基酸序列用于诊断和预防结核感染，诊断试剂盒及其疫苗		
公开(公告)号	US9110061	公开(公告)日	2015-08-18
申请号	US13/811906	申请日	2011-07-25
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当前申请(专利权)人(译)	CELLESTIS有限公司		
[标]发明人	MARIANI FRANCESCA AMICOSANTE MASSIMO COLIZZI VITTORIO SALTINI CESARE		
发明人	MARIANI, FRANCESCA AMICOSANTE, MASSIMO COLIZZI, VITTORIO SALTINI, CESARE		
IPC分类号	G01N33/569 G01N33/53 G01N33/68 A61K38/10 A61K38/16 C07K14/35		
CPC分类号	A61K38/10 A61K38/164 C07K14/35 G01N33/5695 C07K7/08 G01N33/56972 G01N33/6866 G01N2333/57 G01N2333/35 A61P31/04 A61P31/06		
优先权	102010901860436 2010-07-23 IT		
其他公开文献	US20130252260A1		
外部链接	Espacenet USPTO		

摘要(译)

本发明涉及基因序列或其部分的用途，其特征在于其属于结核分枝杆菌目前感染的人巨噬细胞中的体外和离体诱导，抑制或保守基因的种类，并且相应的肽或共有肽或蛋白质，用于制备用于诊断和预防活动性或潜伏性疾病的特异性生物标志物。

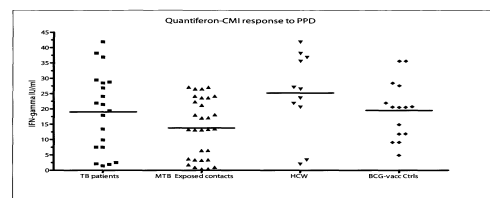


Fig.1

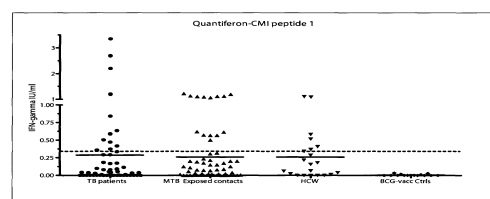


Fig.2