(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
26 January 2006 (26.01.2006)

PCT

(10) International Publication Number
WO 2006/008759 A2

(51) International Patent Classification: Not classified

(21) International Application Number:
PCT/IN2005/000244

(22) International Filing Date: 20 July 2005 (20.07.2005)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
709/CHE/2004 21 July 2004 (21.07.2004) IN

(71) Applicant *(for all designated States except US)*: **CENTRE FOR DNA FINGERPRINTING AND DIAGNOSTICS [CDFD]** [IN/IN]; Department of Biotechnology, Ministry of Science & Technology, Government of India, Nacharam, Hyderabad 500 076 (IN).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **HASNAIN, Seyed, Ehtesham** [IN/IN]; Laboratory of Molecular and Cellular Biology, Centre for DNA Fingerprinting and Diagnostics [CDFD], Nacharam, Hyderabad 500 076 (IN). **CHAKHAIYAR, Prachee** [IN/IN]; Laboratory of Molecular and Cellular Biology, Centre for DNA Fingerprinting and Diagnostics [CDFD], Nacharam, Hyderabad 500 076 (IN). **NAGALAKSHMI, Yellajosyula** [IN/IN]; Mahavir Hospital and Research Centre, Hyderabad 500 004 (IN). **ARUNA, Bandi** [IN/IN]; Laboratory of Molecular and Cellular Biology, Centre for DNA Fingerprinting and Diagnostics [CDFD], Nacharam, Hyderabad 500 076 (IN). **MURTHY, Kollur, J., R.,** [IN/IN]; Mahavir Hospital and Research Centre, Hyderabad 500 004 (IN). **KOTOCH, Vishwa, M.,** [IN/IN]; Laboratory of Microbiology, Central Jalma Institute for Leprosy, Agra 282 001 (IN).

(74) Agents: **BHOLA, Ravi** et al.; K & S PARTNERS, 84-C, C6 LANE OFF CENTRAL AVENUE, SAINIK FARMS, NEW DELHI 110 062 (IN).

(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NA, NG, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, SM, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IS, IT, LT, LU, LV, MC, NL, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Declaration under Rule 4.17:

— *as to the applicant's entitlement to claim the priority of the earlier application (Rule 4.17(iii)) for all designations*

Published:

— *without international search report and to be republished upon receipt of that report*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) **Title:** ANTIGENIC PEPTIDES

(57) **Abstract:** The present invention relates to peptide antigens of SEQ ID Nos. 1 to 10; repeat motif Gly-X-Gly-Asn-X-Gly of SEQ ID No. 11; and a method of developing drug against tuberculosis, said method comprising steps of targeting the proposed drug towards peptide antigens of SEQ ID Nos. 1 to 11, and thereby developing the drug against tuberculosis.

WO 2006/008759 A2

ANTIGENIC PEPTIDES

FILED OF THE INVENTION

The present invention relates to peptide antigens of SEQ ID Nos. 1 to 10; repeat motif Gly-X-Gly-Asn-X-Gly of SEQ ID No. 11; and a method of developing drug against tuberculosis, said method comprising steps of targeting the proposed drug towards peptide antigens of SEQ ID Nos. 1 to 11, and thereby developing the drug against tuberculosis.

INTRODUCTION

The existence of PE/PPE gene families was evident even before the *Mycobacterium tuberculosis* genome was sequenced with occasional reports of occurrence of glycine and alanine rich multiple repetitive sequences in the genome [1] or the identification of a few fibronectin binding proteins [2]. Sequencing categorized the PE/PPE gene families as two large unrelated families of highly acidic glycine rich proteins that constitute about 10% of the coding capacity of the genome [3]. Comparative genome sequencing in various mycobacterial species revealed that by and large PE and PPE gene families are unique to *Mycobacterium tuberculosis* with few homologues in *M. leprae, M. bovis, M. marinum etc* [4]. Amongst the *M. leprae* homologues, a major serine rich antigen is expressed in leprosy patients [5].

It is generally believed that the PE and PPE genes could be a source of antigenic variability. A recombinant PE_PGRS (Rv1759c) protein was shown to possess fibronectin binding properties and was also recognized by patient area [6]. The same group also reported immense intra-strain variability in the PGRS domain with the N-terminal region staying constant. Transposon insertion in the PE_PGRS gene (Rv1818c) was shown to reduce macrophage infection ability of *Mtb* [7]. Surface localization of a PPE protein (Rv1917c) and many other PE_PGRS proteins has been reported [8.9]. Few PE_PGRS genes have also been shown to be expressed during preclinical infection [10]. Dissection of the PE_PGRS genes into PE and the PGRS domains to study their specific immunological response during mice infection revealed that the PE region can elicit an effective cellular immune response and the humoral response is largely directed against the Gly-Ala rich PGRS domain [11]. The involvement of PE/PPE genes in the viluence

of the pathogen has also been reported [12]. We recently described the biophysical and biochemical properties of a PPE gene, Rv2430c and further showed that it is an immunodominant antigen of *Mtb* [13,14,15].

In this study, we used an *in-silico* approach to identify probable antigens from the PPE_MPTR (Major Polymorphic Tandem Repeat) subfamily and studied the humoral and cellular immune response to the same using well characterized patient samples. Synthetic peptides corresponding to regions of high antigenic index of the protein were used to map the antigenic domains and assess the antigenic potential of the Gly-X-Gly-Asn-X-Gly repeat motif in eliciting a differential immune response. Our results suggest that the PPE_MPTR ORF Rv2608 could be involved in directing the host towards development of a more humoral type of immune response.

MATERIALS AND METHODS

PCR-RFLP analysis of the PPE ORF, Rv2608:

PCR-RFLP was carried out to examine if Rv2608 exhibited polymorphism in different clinical isolates of *M. tubercuolsis*. Briefly, Rv2608 was PCR amplified from about 30 different clinical isolates and the amplified product was digested with *Sau*3AI enzyme. The digested product was separated on a 10% polyacrylamide gel and visualized under UV after ethidium bromide staining.

Cloning, overexpression and purification of Rv2608, a PPE MPTR subfamily member of *M. tuberculosis*:

The PPE ORF, Rv2608 was amplified from *M. tuberculosis* H37Rv genomic DNA using primers carrying specific restriction enzyme sites to enable directional cloning. The amplified gene was first cloned in pGEMT easy vector followed by subcloning in

pRSETa expression vector. Expression of the 59.6kDa recombinant Rv2608 protein in *E. coli* BL21 cells was achieved as described earlier [14]. The recombinant protein was purified to homogeneity on a Nickel NTA affinity column (Qiagen Inc).

Synthetic peptides: The PPE ORF, Rv2608 was scanned to identify regions of high antigenic index using the Protean software of Lasergene Navigator™ (DNA STAR). Ten synthetic peptides of varying lengths corresponding to *in-silico* predicted regions of high antigenic index were commercially obtained as lyophilized powders. Peptide stocks of concentration 0.1 mg/ml were prepared in carbonate bicarbonate buffer and stored in aliquots at -70^0C.

Subjects: Fifty one TB patients confirmed by tuberculin skin test, radiographic examination and observation of Acid Fast Bacilli (AFB) in sputum for pulmonary TB and at the site of presumed TB in case of extrapulmonary infection were selected for this study. These patients were reporting to the Out Patient Department of the Mahavir Hospital and Research Centre Hyderabad, India. All the patients with confirmed diagnosis of TB were culture positive as well. We categorized the patients as follows: Category I: Individuals (n=22) diagnosed for TB for the first time; Category II: Individuals (n=21) with a relapsed TB and Category III: Extrapulmonary TB patients (n=8). Sera were collected from all the subjects during early stage of infection when chemotherapy had just started. Healthy control (n=10) sera were taken from the laboratory staff of CDFD. These were individuals who had not had a prolonged direct contact with a TB patient. As this study was carried on a PPE gene family member of *Mtb*, members of which are unique to mycobacteria [3], cross reactivity to this protein

would not be expected and therefore control subjects with other bacterial infection were not considered necessary for inclusion in our study.

ELISA and Lymphocyte proliferation assay:

All the 51 patients were tested against each of the 10 peptide antigens to evaluate for a B cell as well as a T cell response. ELISAs with 2μg/ml of rHsp10/rRv2608 protein /synthetic peptides were carried out as described earlier [14]. The Lymphocyte proliferation assays were carried out essentially as per method described earlier with a few modifications [16]. Heparinised blood was drawn and diluted with equal volume of RPMI1640 medium without serum. Diluted blood was layered on Ficoll gradient in 1:3 proportion. After a low speed (800g) centrifugation for 30 minutes, the peripheral blood mononuclear cells (PBMCs) were isolated and washed twice for 10 minutes at 800g to remove the cell debris and platelets. Cell concentration was adjusted to 10^6/ml. Viability of the cells was checked using Trypan Blue. To each well of the microtiter plates, 0.1ml of cell suspension and 0.1 ml of antigen (2μg/ml) was added. ConA (Concanavalin A) was used as a positive control antigen. Control and experimental cultures were run in triplicate. The plate was incubated at 37^0C with 5% CO_2 for a period of 72 hours. At the end of the 3rd day, 15μl of the tetrazolium salt MTT (2mg/ml) was added and incubated for another 4 hours. The culture was terminated and the MTT crystals were dissolved in 100 μl of acidified isopropanol. After one hour, the optical density was recorded using ELISA plate reader at a dual wavelength of 570 nm and 620 nm reference filter. Data were expressed as Stimulation Index (S.I.) i.e. ratio of the mean O.D. of experimental cultures (with test antigen) to the mean O.D. of control cultures (without antigen). S.I. greater than or equal to 2 was considered as positive stimulation index.

.Statistical methods:

Analysis of variance (ANOVA) as a test of statistical significance was performed using an online software (http://www.physics.csbsju.edu/stats/ANOVA.html) to calculate the p values and determine if there was any difference between different patient categories with respect to each antigen tested. The 95% confidence intervals for means were also determined for each set of data. Differences between groups were considered statistically significant if the 95% confidence interval limits did not overlap. To ascertain the results obtained by ANOVA, we also carried out Kruskal Wallis non-parametric test (http://department.obg.cuhk.edu.hk/ResearchSupport/KruskalIWallis.ASP). Additionally, we also carried out t tests for paired comparison of means. $p<0.05$ was considered statistically significant.

RESULTS:

Genetic variation in the PPE ORF, Rv2608

PE/PPE genes are predicted to be a source of antigenic variability of *M. tuberculosis* and polymorphism in a few of them based upon variation in the number of repeat sequences has already been reported [6]. We analyzed the PPE gene, Rv2608 of the major polymorphic tandem repeat (MPTR) subclass by PCR-RFLP to score for the presence of genetic variation in different clinical isolates. The 1.7kb amplicon was digested with *Sau*3AI and the digest was electrophoresed on a 10% polyacrylamide gel. 16% of the clinical isolates showed a deviation from the normal band pattern. Figure 1 gives the complete summary of the polymorphism obtained in 30 different clinical isolates. The disappearance of restriction fragments was restricted to the C terminus of the ORF, which is the predicted variable region of the PPE ORFs. It was therefore important to further

evaluate Rv2608 in terms of its ability to elicit B and T cell response in order to study its role as a possible antigen for immune surveillance.

Expression and purification of the rPPE protein:

To evaluate the antigenic ability of Rv2608, the corresponding gene was expressed in *E. coli* BL21 cells and purified as a 6X His-tag fusion protein. Purified recombinant Rv2608 was fractionated by electrophoresis on a 12% polyacrylamide gel. A single band corresponding to 59.6kDa protein was observed upon staining the gel with Coomassie Brilliant Blue dye (Figure 2). The expression of the gene was confirmed by probing the membrane containing the total cellular protein of *E. coli* BL21 cells harboring the Rv2608 construct with anti-Histidine antibody. There was no leaky expression of the protein in uninduced cells. The recombinant protein was largely present in the insoluble fraction and was therefore purified in the presence of 8M urea (Figure 2, LaneE). The yield of the protein was 6mg/litre of culture. The recombinant protein was dialyzed overnight and used for immunoreactivity analysis.

Design of synthetic peptides based on antigenicity prediction of Rv2608:

In-silico analysis of Rv2608 revealed the presence of two regions of high antigenicity: Two amino acid stretches (37 amino acids and 25 amino acids) corresponding to important antigenic epitopes within Rv2608 were selected for peptide synthesis (Figure 3a). Additional eight overlapping regions (Figure 3b) which were essentially the subsets of the two main peptides were also selected for peptide synthesis. These peptides were used to map the antigenic domains of the protein. Table 1 shows the amino acid sequences of all the 10 synthetic peptides used in the present study. The peptides were part of the C terminal region of Rv2608 and apart from the high antigenic index also

possessed the repeat motif Gly-X-Gly-Asn-X-Gly, characteristic of the PPE_MPTR gene family.

rPPE protein shows positive reactivity to sera from different categories of TB patients:

The humoral response to the recombinant PPE protein was characterized by measuring serum IgG antibodies to the protein using ELISA. Antibody response was analyzed as a function of mean absorbance at 492nm. Recombinant Hsp10, a major antigen of *M. tuberculosis* was used for comparison of the response to the rPPE protein. It was observed that for all the patient categories, serum reactivity to rRv2608 was equal to or higher than the response to Hsp10 ($p>0.05$, indicating no difference between the response to HSP10 and Rv2608) (Figure 4). Healthy controls also showed some reactivity to the recombinant protein, however the response however was significantly less when compared to that of patients ($p=0.0002$ using student's t test as a test of statistical significance for paired comparison of means between the patients and healthy controls).

Synthetic peptides corresponding to regions of high antigenicity elicit strong humoral immune response in patients with relapsed TB infection:

Having shown that the recombinant protein coded by Rv2608 elicited an antibody response which was equal to or higher than that elicited by Hsp10 antigen in all the categories of TB patients selected for the study, we tried to dissect differential responses if any as a function of patient category. For this, synthetic peptides spanning the two major antigenic regions within Rv2608 (P1 and P2) were used in ELISA (Table i). The results suggest that these peptides strongly react with patient sera (Figure 5) and hence the protein must be generating a strong humoral response in the host. Since a positive

response was obtained with the peptides 1 and 2, patient sera were also tested for reactivity against the short overlapping peptide sequences 1a, 1b, 1c, 1d, 1e which were all components of peptide 1 and 2a, 2b and 2c which were a part of peptide 2. The results obtained indicate that even these overlapping peptide stretches react equally well with patient sera. Exact mapping of the antigenic region was not possible as most of the peptides showed a similar response. This was a reflection of the fact that the Gly-X-Gly-Asn-X-Gly repeat motifs were present in all the peptides. Very interestingly, there was a significantly varied response to the peptides in different category of TB patients which was not so when the complete recombinant Rv2608 protein was used. The peptides could clearly distinguish between the patient categories ($p<0.001$ using ANOVA for each peptide antigen) (Table 2). While humoral response observed in case of fresh infection cases (Category I) was similar to that of extrapulmonary TB patients (Category III), category II or the relapsed cases showed an unusually high antibody response to all the peptides. The response of Category II patients was significantly higher than Category I or III ($p< 0.001$ for both, using t test as a test of statistical significance).

The T cell response of TB patients to Rv2608 peptide antigens was low and the differences between various categories of patients were not evident:

T cell proliferation assays were carried out to evaluate the response to different synthetic peptides. The overall T cell response of patients to these peptides was very low (S.I.<2) and the response could not distinguish between patient categories ($p>0.05$, using ANOVA and Kruskal Wallis test) at least for peptide 1 and its derivatives.

Peptide 2 and its derivatives exhibited a higher response in fresh infection cases as against relapsed and extrapulmonary cases ($p<0.05$ for both, using t test for paired

comparison of means). As can be noted from the amino acid sequence of the peptides (Table 1), peptide 2 has lesser number of glycine asparagine repeats and shows a higher T cell proliferative response in fresh infection cases.

DISCUSSION:

The ORF Rv2608 selected for the present study is a member of the PPE_MPTR class which is characterized by the presence of a conserved N-terminal region and a C-terminal domain with major polymorphic tandem repeats (MPTR) of Gly-X-Gly-Asn-X-Gly residues. Apart from this, the ORF also possesses regions of high antigenic index, which is a measure of overall hydrophilicity and surface probability. To test if polymorphism of the C-terminal region of this ORF exists in different clinical isolates of *M. tuberculosis*, PCR amplified Rv2608 was subjected to PCR-RFLP analysis. The observed variation in the band pattern lends weight to the hypothesis that PE/PPE genes, notably Rv2608 are perhaps a source of antigenic variability in the otherwise conserved genome of *M. tuberculosis*.

The rRv2608 protein was used in ELISAs to determine its reactivity to patient sera. The primordial observation that the recombinant protein reacted with patient sera indicates that this protein is definitely expressed during infection. Serum response of patients as well as healthy controls to rRv2608 was equivalent to or greater than the response to Hsp10, a well documented antigen of *Mtb* [17]. While category wise differentiation of serum reactivity towards the full length recombinant protein was not very apparent, it was significant to note that the extrapulmonary TB patients showed less reactivity with rRv2608 protein as compared to Category I or II ($p=0.048$). It will be worthwhile to

explore whether Rv2608 represents a protein(s) required by the bacterium to establish a pulmonary infection.

Since the serum response to the recombinant PPE protein was equal to or greater than Hsp10, it was decided to possibly map the antigenic domains of the probable PPE antigen using a synthetic peptide approach [18-20]. Peptides corresponding to regions of high antigenic index were accordingly designed. Our analyses of the comparative humoral immune responses indicate that the serum response of patients to all the ten peptides is similar. This could be explained by the fact that all the peptides have a common repeat motif thereby eliciting similar response. While this negated our efforts to map the immunodominant epitope required for eliciting a strong humoral immune, a difference in the response of patients categorized according to different states of infection was surprisingly evident. Category II patients (relapsed infection cases) demonstrate the highest B cell response to the peptides followed by extrapulmonary TB cases.

The synthetic peptides were also used for T cell proliferation assays with the peripheral blood mononuclear cells of all category patients. It has been earlier shown that in about 90% of patients with active TB, there is a significant antibody response and/or T cell proliferative response to peptide specific single antigens of *Mtb* [21]. The 38kDa antigenic protein of *M tuberculosis* is a potent stimulus for both T cell and B cell responses in humans [22,23]. The T cell proliferative response to the synthetic peptides was of the order of fresh infection cases> relapsed TB > extrapulmonary TB cases at least for peptide 2 and its derivatives. However, the observed Stimulation Index (SI) with all the peptides was very low in all categories of TB patients (S.I.<2). A high humoral response and a low cellular immune response to the peptides in category II patients points

to an important possible function of the PE/PPE gene families. It is likely that these antigens play a role in evading the host immune response and prevent the establishment of an effective cellular response, which is required to contain the disease. The positive T cell response in some cases could be explained by the fact that IgG antibody responses again require the involvement of helper T cells.

Antibody levels usually decrease in cured TB cases but dramatically increase in patients showing poor compliance [24]. High antibody response to the peptides and a low T cell response hence explain the relapse of infection in category II patients. In vivo, it is possible that the responsive T cells are not able to expand as the glycine, asparagine repeat motifs somehow prevent antigen processing. The situation can be equated with the Epstein Barr Virus Nuclear antigen, where again the Gly-Ala repeat regions play an important role in preventing antigen processing [25]. Peptides 2 and 2c, which have lesser number of Gly-Asn repeats show a comparatively higher T cell response.

In conclusion, we have been able to establish a relationship between immune responses to the PPE antigen and the status of the disease (fresh or relapsed TB). The present study is the first report wherein we demonstrate, in a clinical setting, that the repeat sequences present within Rv2608 elicit a high humoral immune response and a low T cell response. Since PPE_MPTR is a gene family of *Mtb* of which Rv2608 is a member sharing the MPTR motif, it is likely that other members of the same family may also serve the same function in the bacterium. Our data contribute towards a better understanding of humoral as well as cellular immune responses elicited by PPE antigens. The practical utility of using these peptides for differentiating fresh infection from relapsed or reactivation cases is another interesting proposition.

REFERENCES

1. Poulet S and Cole ST. Characterization of the highly abundant polymorphic GC-rich-repetitive sequence (PGRS) present in *Mycobacterium tuberculosis*. Arch Microbiol **1995**; 163:87-95.
2. Abou-Zeid C, Garbe T, Lathigra R, Wiker HG, Harboe M, Rook GA, Young DB. Genetic and immunological analysis of *Mycobacterium tuberculosis* fibronectin-binding proteins. Infect Immun **1991**; 59:2712-8.
3. Cole ST, Brosch R, Parkhill J, Garnier T *et al.* Deciphering the biology of *Mycobacterium tuberculosis* from the complete genome sequence. Nature **1998**; **393**:537-44.
4. Cole ST. Comparative and functional genomics of the *Mycobacterium tuberculosis* complex. Microbiology **2002**; 148:2919-28.
5. Vega-Lopez F, Brooks LA, Dockrell HM, De Smet KA, Thompson JK, Hussain R, Stoker NG. Sequence and immunological characterization of a serine-rich antigen from *Mycobacterium leprae*. Infect Immun **1993**; 61:2145-53.
6. Espitia C, Laclette JP, Mondragon-Palomino M, Amador A, Campuzano J, Martens A, Singh M, Cicero R, Zhang Y, Moreno C. The PE-PGRS glycine-rich proteins of *Mycobacterium tuberculosis*: a new family of fibronectin-binding proteins? Microbiology **1999**; 145:3487-95.
7. Brennan MJ, Delogu G, Chen Y, Bardarov S, Kriakov J, Alavi M, Jacobs WR Jr. Evidence that mycobacterial PE_PGRS proteins are cell surface constituents that influence interactions with other cells. Infect Immun **2001**; 69:7326-33.

8. Sampson SL, Lukey P, Warren RM, van Helden PD, Richardson M, Everett MJ. 2001. Expression, characterization and subcellular localization of the *Mycobacterium tuberculosis* PPE gene Rv1917c. Tuberculosis (Edinb) **2001**; 81:305-17.

9. Banu S, Honore N, Saint-Joanis B, Philpott D, Prevost MC, Cole ST. 2002. Are the PE-PGRS proteins of *Mycobacterium tuberculosis* variable surface antigens? Mol Microbiol **2002**; 44:9-19.

10. Singh KK, Zhang X, Patibandla AS, Chien P Jr, Laal S. Antigens of *Mycobacterium tuberculosis* expressed during preclinical tuberculosis: serological immunodominance of proteins with repetitive amino acid sequences. Infect Immun **2001**; 69:4185-91.

11. Delogu G, Brennan MJ. Comparative immune response to PE and PE_PGRS antigens of *Mycobacterium tuberculosis.* Infect Immun **2001**; 69:5606-11.

12. Ramakrishnan L, Federspiel NA, Falkow S. Granuloma-specific expression of Mycobacterium virulence proteins from the glycine-rich PE-PGRS family. Science **2000**; 288:1436-9.

13. Choudhary RK, Pullakhandam R, Ehtesham NZ, and Hasnain SE. Expression and characterization of Rv2430c, a novel immunodominant antigen of *Mycobacterium tuberculosis.* Protein Expresion and Purification **2004**; 00:00-000 (In Press).

14. Choudhary RKC, Mukhopadhyay S, Chakhaiyar P, Sharma N, Murthy KJR, Katoch VM and Hasnain SE. PPE antigen Rv2430c of *Mycobacterium tuberculosis* induces a strong B cell response. Infect Immun **2003**; 71: 6338-43.

15. Chakhaiyar P and Hasnain SE. Defining the mandate of tuberculosis research in a post genomic era. Medical Principles and Practice **2004**; 00:00-000 (In Press).

16. Van de Loosdrecht AA, Beelen RH, Ossenkoppele GJ, Broekhoven MG, Langenhuijsen MM. A tetrazolium-based colorimetric MTT assay to quantitate human monocyte mediated cytotoxicity against leukemic cells from cell lines and patients with acute myeloid leukemia. J Immunol Methods **1994**; 174:311-20.

17. Young DB, Garbe TR. Heat shock proteins and antigens of *Mycobacterium tuberculosis*. Infect Immun **1991**; 59:3086-93.

18. Miguez J, Laferte J, Tejero Y, Gonzalez G, Otero AJ, Rivero J, Duarte C. Evaluation of the serologic response against two consensus V3 loop peptides from human immunodeficiency virus-1 in Cuban patients. Int J Infect Dis **1998**; 2:221-5.

19. Liljeqvist JA, Trybala E, Svennerholm B, Jeansson S, Sjogren-Jansson E, Bergstrom T. Localization of type-specific epitopes of herpes simplex virus type 2 glycoprotein G recognized by human and mouse antibodies. J Gen Virol **1998**; 79:1215-24.

20. Benitez J, Palenzuela D, Rivero J, Gavilondo JV. A recombinant protein based immunoassay for the combined detection of antibodies to HIV-1, HIV-2 and HTLV-I. J Virol Methods **1998**; 70:85-9.

21. Wilkinson RJ, Vordermeier HM, Wilkinson KA, Sjolund A, Moreno C, Pasvol G, Ivanyi J. Peptide-specific T cell response to *Mycobacterium tuberculosis*: clinical spectrum, compartmentalization, and effect of chemotherapy. J Infect Dis **1998**; 178:760-8.

22. Young D, Kent L, Rees A, Lamb J, Ivanyi J. Immunological activity of a 38-kilodalton protein purified from *Mycobacterium tuberculosis*. Infect Immun **1986**; 54:177-83.

23. Andersen AB, Hansen EB. Structure and mapping of antigenic domains of protein antigen b, a 38,000-molecular-weight protein of *Mycobacterium tuberculosis*. Infect Immun **1989**; 57:2481-8.

24. Bothamley GH, Schreuder GM, de Vries RR, Ivanyi J. Association of antibody responses to the 19-kDa antigen of *Mycobacterium tuberculosis* and the HLA-DQ locus. J Infect Dis **1993**; 167:992-3.

25. Levitskaya J, Coram M, Levitsky V, Imreh S, Steigerwald-Mullen PM, Klein G, Kurilla MG, Masucci MG. Inhibition of antigen processing by the internal repeat region of the Epstein-Barr virus nuclear antigen. Nature **1995**; 375:685-8.

DETAILED DESCRIPTION OF THE INVENTION

Accordingly the present invention relates to a peptide antigens of SEQ ID Nos. 1 to 10.

In yet another embodiment of the present invention, wherein the repeat motif elicits high humoral immune response and low T-cell response.

In still another embodiment of the present invention, wherein a repeat motif Gly-X-Gly-Asn-X-Gly of SEQ ID No. 11.

In yet another embodiment of the present invention, wherein the repeat motif elicits high humoral immune response and low T-cell response.

In still another embodiment of the present invention, wherein "X" represents any amino acid residue.

One more embodiment of the present invention, wherein a method of developing drug against tuberculosis, said method comprising steps of:

a. targeting the proposed drug towards peptide antigens of SEQ ID Nos. 1 to 11, and

b. developing the drug against tuberculosis.

Table1: Amino acid sequence of the synthetic peptides

	AMINO ACID SEQUENCE*
P1	DNIGNANIGFGNRGDANIGIGNIGDRNLGIG NTGNWK (37)
P2	RPGLDELSFTLTGNPNRPDGGILTK (25)
P1a	DNIGNANIGFGNK (13)
P1b	NIGFGNRGDANIK (13)
P1c	RGDANIGIGNIGK (13)
P1d	GIGNIGDRNLGIK(13)
P1e	DRNLGIGNTGNWK (13)
P2a	RPGLDELSFTLTK (13)
P2b	LSFTLTGNPNRPK (13)
P2c	GNPNRPDGGILTK (13)

*Residues in bold represent the Glycine-Asparagine repeat motifs

Table2: Summary of the results of statistical analyses to estimate differences in humoral immune response to different peptide antigens

Peptide Antigen	Mean (O.D. at 492nm)	95% confidence interval of Mean	Degree of freedom	F value	P value	Difference between categories (Significant (S) / Not Significant
P1 I	0.412	0.338 to 0.486	2	12.69	<0.0001	S
II	0.675	0.598 to 0.750				
III	0.483	0.359 to 0.606				
P1a I	0.426	0.360 to 0.492	2	29.69	<0.0001	S
II	0.770	0.704 to 0.836				
III	0.473	0.363 to 0.582				
P1b I	0.469	0.416 to 0.521	2	35.47	<0.0001	S
II	0.775	0.720 to 0.828				
III	0.520	0.432 to 0.607				
P1c I	0.416	0.364 to 0.467	2	70.48	<0.0001	S
II	0.810	0.756 to 0.862				
III	0.380	0.293 to 0.464				
P1d I	0.482	0.427 to 0.535	2	33.47	<0.0001	S
II	0.787	0.731 to 0.842				
III	0.527	0.437 to 0.617				
P1e I	0.407	0.348 to 0.464	2	31.26	<0.0001	S
II	0.711	0.651 to.0.770				
III	0.405	0.308 to 0.501				
P2 I	0.480	0.413 to 0.546	2	14.51	<0.0001	S
II	0.706	0.637 to 0.774				
III	0.441	0.331 to 0.551				
P2a I	0.473	0.412 to 0.533	2	18.63	<0.0001	S

	II	0.689	0.627 to 0.750			
	III	0.392	0.291 to 0.491			
P2b	I	0.450	0.379 to 0.521	2 0.7688	0.4	NS
	II	0.498	0.425 to 0.570			
	III	0.422	0.304 to 0.540			
P2c	I	0.491	0.433 to 0.548	2 10.05	0.0002	S
	II	0.632	0.572 to 0.690			
	III	0.410	0.314 to 0.505			

FIGURE LEGENDS

Figure 1: *Sau*3AI Restriction map of PPE ORF, Rv2608. Arrowheads point to the *Sau*3A sites in the 1743bp ORF. Numbers above the line indicate the size of the restriction fragments (in base pairs) generated after *Sau*3AI digestion. **B**: Summary of *Sau*3AI PCR-RFLP pattern of 30 different clinical isolates of *Mycobacterium tuberculosis.*

Figure 2: Expression and purification of *M. tuberculosis* protein corresponding to the PPE ORF Rv2608. The left panel shows the uninduced and induced cell lysates and proteinmolecular size marker (Lanes 1, 2, M). The right panel shows the purified recombinant protein (Lane 3) and the protein molecular size marker (M).

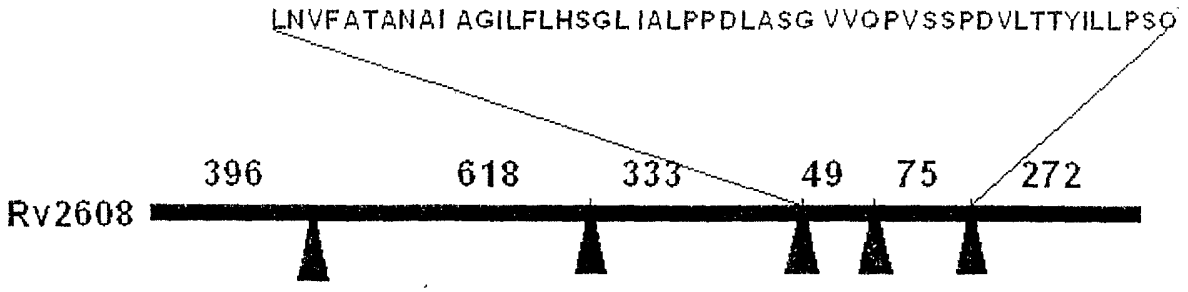
Figure 3: *In-silico* analysis of Rv2608 reveals regions of high antigenic index (potential antigenic determinants). Overall antigenic index of the protein was calculated using the James Wolfinson method of the Protean software of Lasergene Navigator™. The boxed areas indicate the regions selected for designing synthetic peptides to map the region that was actually eliciting a variable immune response. As can be seen, one of the peptides (37mer) is largely composed of Gly-Asn repeats which is lesser in number in the other peptide (23mer). **B**: Stretches of overlapping peptides used for ELISA and T cell proliferation assay. These peptides were used to further map the region that was antigenic.

Figure 4: Antibody response of different categories of TB patients to rRv2608 is equivalent to the response to rHsp10, a well documented antigen of *M. tuberculosis*. Serum reactivity was measured by ELISA and the graph was plotted as patient response (O.D. at 492nm) to rHsp10 and rRv2608. The difference between patient response to Hsp10 and rRv2608 was not significant for all patient categories ($p>0.05$ using paired t tests). However, the response of healthy controls was lower and differed significantly from the patients ($p=0.0002$ using paired t test). (HC= Healthy Controls, Cat= Category)

Figure 5: Antibody response of different categories of TB patients to different synthetic peptides (regions of high antigenicity within Rv2608) as determined by ELISA. Response to all the peptides was plotted as absorbance at 492nm (mean ± SD). The response of Category II patients was significantly higher than Category I or III ($p< 0.001$ for both, using paired t tests) with respect to each peptide antigen.

Claims:

1. Peptide antigens of SEQ ID Nos. 1 to 10.
2. Peptide antigens as claimed in claim 1, wherein the repeat motif elicits high humoral immune response and low T-cell response.
3. A repeat motif Gly-X-Gly-Asn-X-Gly of SEQ ID No. 11.
4. A repeat motif as claimed in claim 3, wherein the repeat motif elicits high humoral immune response and low T-cell response.
5. A repeat motif as claimed in claim 3, wherein "X" represents any amino acid residue.
6. A method of developing drug against tuberculosis, said method comprising steps of:
    a. targeting the proposed drug towards peptide antigens of SEQ ID Nos. 1 to 11, and
    b. developing the drug against tuberculosis.



A

Location of *Sau*3AI sites	Expected bands (bp)	Additional bands (bp)	25 isolates	2 isolates	3 isolates
396	396		+	+	+
1014	618		+	+	+
1347	333		+	+	+
1396	49		+	+	-
1471	75		+	-	-
1753	272		+	+	+
		~120	-	+	+
		~100	-	+	+

B

Figure 1

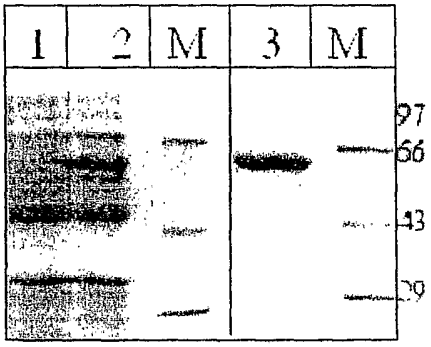
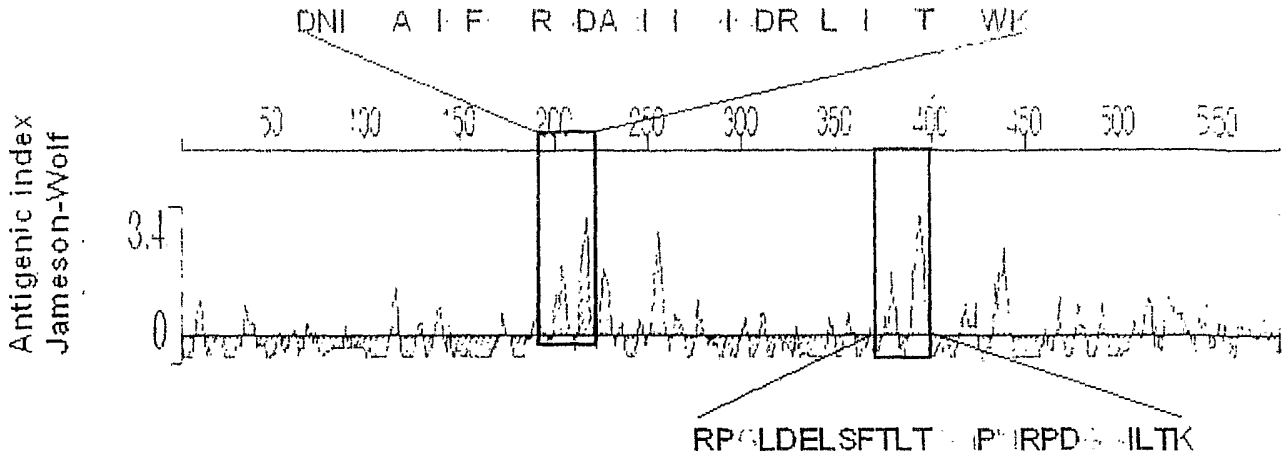
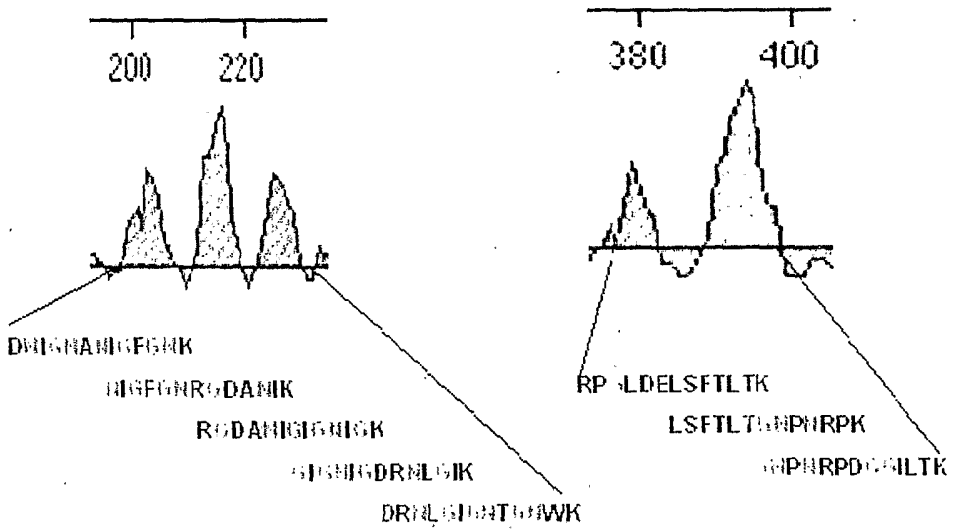


Figure 2


Antigenic index
Jameson-Wolf
3.4
0
50
100
150
200
250
300
350
400
450
500
550

A


200
220
380
400

B

Figure 3

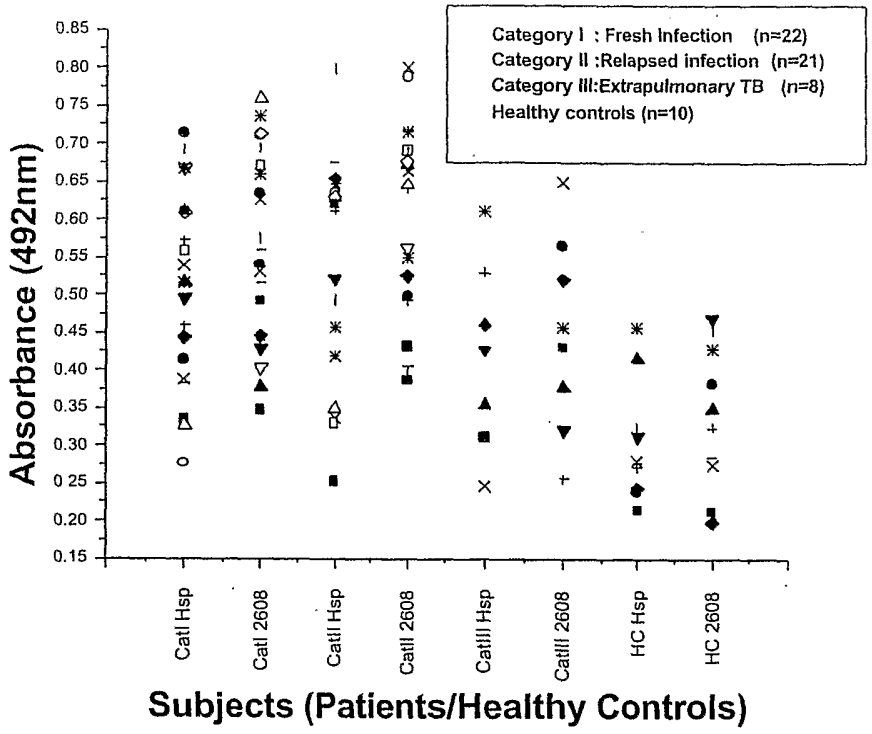

Category I : Fresh Infection (n=22)
Category II :Relapsed infection (n=21)
Category III:Extrapulmonary TB (n=8)
Healthy controls (n=10)
Absorbance (492nm)
0.85
0.80
0.75
0.70
0.65
0.60
0.55
0.50
0.45
0.40
0.35
0.30
0.25
0.20
0.15
CatI Hsp
CatI 2608
CatII Hsp
CatII 2608
CatIII Hsp
CatIII 2608
HC Hsp
HC 2608
Subjects (Patients/Healthy Controls)

Figure 4

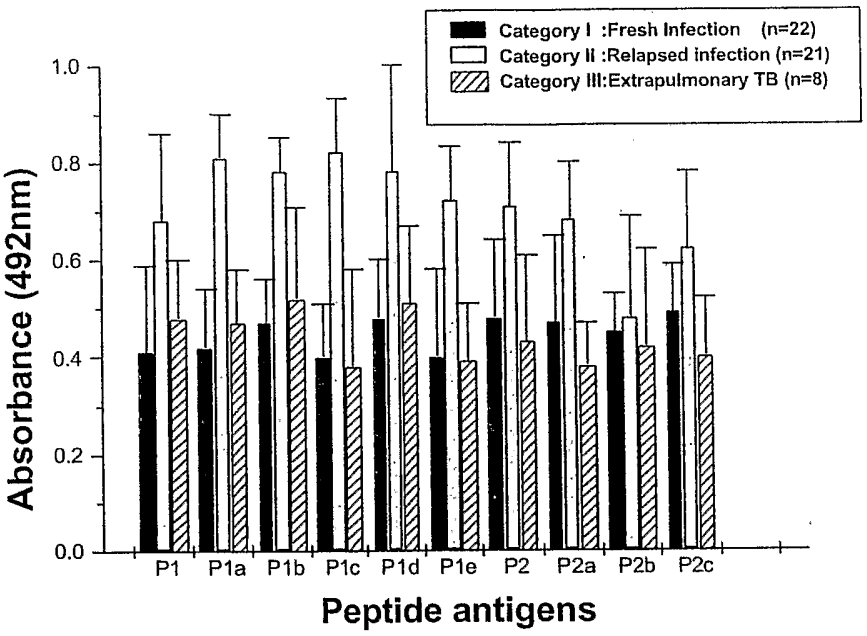

Category I :Fresh Infection (n=22)
Category II :Relapsed infection (n=21)
Category III:Extrapulmonary TB (n=8)
Absorbance (492nm)
1.0
0.8
0.6
0.4
0.2
0.0
P1
P1a
P1b
P1c
P1d
P1e
P2
P2a
P2b
P2c
Peptide antigens

Figure 5