The RD1-encoded antigen Rv3872 of *Mycobacterium tuberculosis* as a potential candidate for serodiagnosis of tuberculosis

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ABSTRACT

Tuberculosis (TB) infections in India account for one-third of the global burden, making it important to develop speedy, cost-effective diagnostic tools. This study evaluated recombinant RD1-encoded antigens of *Mycobacterium tuberculosis* as tools for serodiagnosis by determining the immunological reactivity of these proteins against sera from healthy, bacille Calmette–Guerin (BCG)-vaccinated and TB-infected individuals from Kolkata. Rv3872, Rv3875 (ESAT-6) and Rv3878 were able to discriminate healthy BCG-vaccinated controls from TB patients. Rv3872 showed the highest level of antibody response in comparison with other antigens, and also showed statistically significant differences between pulmonary (p < 0.0001) or extra-pulmonary (p < 0.001) TB patients and healthy BCG-vaccinated individuals. The levels of antibody were measured using 20-mer overlapping peptides spanning the entire Rv3872 sequence. The immunological reactivity against a mixture of two peptides (P8 and P9) encompassing amino-acids 57–84 correlated well with that obtained using full-length Rv3872. This result was explained by the fact that two of the predicted regions of high antigenicity lie within amino-acid residues 57–85 of Rv3872. The high sensitivity and specificity of Rv3872, as well as the mixture of two synthetic overlapping peptides derived from Rv3872, highlight their potential and argue in favour of their use in serodiagnosis of both pulmonary and extra-pulmonary TB.

Keywords Antigen, diagnosis, ELISA, *Mycobacterium tuberculosis*, RD1-encoded antigen, serodiagnosis

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INTRODUCTION

India is classified, along with the sub-Saharan African countries, as a country with a high burden of tuberculosis (TB), accounting for one-third of the total global burden of this disease. Estimating the burden of the disease in India, Gopi *et al.* [1] reported 3.8 million cases of bacillary (either sputum/smear- or culture-positive) pulmonary TB and 0.8 million cases of extra-pulmonary TB. Nearly 2 million Indian citizens develop TB each year, and one in three Indian citizens carries *Mycobacterium tuberculosis*. According to the Indian Ministry of Health and Family Welfare (http://tbcindia.org), an individual in India dies every minute from TB, with 75% being aged between 15 and 54 years. Diagnosis still depends largely on radiography, sputum microscopy and bacterial culture, often leading to delays in treatment. This underscores the need to develop robust, cost-effective diagnostic tools.

ELISA-based serological tests to detect antibodies to *M. tuberculosis* components in clinical specimens have the potential of providing inexpensive and robust tools for diagnosis of active TB under the conditions encountered commonly in developing countries such as India. Although a number of seroantigens have been identified in recent years, these are of low sensitivity and specificity, because of, in part, the heterogeneity of the antibody response in TB patients from different geographical locations [2,3]. Previous studies have explored the utility of antigens encoded by a 9.5-kb genomic DNA region of *M. tuberculosis*, designated RD1, that is present in all virulent and
clinical strains of *M. tuberculosis* and *Mycobacterium bovis* [4,5], but deleted in all *M. bovis* bacille Calmette–Guérin (BCG) vaccine strains. The present study expressed and evaluated some of the RD1-encoded antigens of *M. tuberculosis*, and evaluated their usefulness as tools for serodiagnosis by determining the immunological reactivity of these proteins with sera from healthy BCG-vaccinated and TB-infected individuals from Kolkata.

**MATERIALS AND METHODS**

**Study population**

All samples were obtained for routine diagnostic analysis from patients (who had given informed consent) who were reporting to the Bengal Tuberculosis Association for directly observed short-course therapy, and from healthy BCG-vaccinated individuals, in accordance with the guidelines of the Institutional Ethics Committee. Healthy donors were individuals without prolonged direct contact with TB patients, who had been vaccinated with *M. bovis* BCG >10 years before reporting to the clinic.

The study population (n = 542) was divided into the following groups: group 1 (n = 240) comprised patients diagnosed with pulmonary TB (as confirmed by examination of sputum for acid-fast bacilli and/or culturing) for the first time, with no history of treatment for TB; group 2 (n = 179) comprised patients with extra-pulmonary TB, as diagnosed by clinical and radiographical findings, histopathological examination and culture of biopsy samples; and group 3 (n = 123) comprised healthy BCG-vaccinated donors. Patients with both pulmonary and extra-pulmonary manifestations, as well as those with tuberculous meningitis, were excluded from the study. Sera were collected from all patients before chemotherapy was commenced.

**Molecular biological procedures**

Standard procedures for cloning and analysis of DNA, PCR and transformation were used [6]. Enzymes used to manipulate DNA were from Roche Applied Sciences (Mannheim, Germany). All constructs made by PCR were sequenced to verify their integrity. Genomic DNA of *M. tuberculosis* strain H37Rv was a generous gift from J. Tyagi (All India Institute of Medical Sciences, New Delhi, India). The genes encoding Rv3872, Rv3873, Rv3874 (CFP-10) and Rv3878 were amplified from the genomic DNA of *M. tuberculosis* H37Rv using the sense and antisense primers shown in Table 1. Rv3875 (ESAT-6), cloned in pET2b, was obtained from J. Belisle (Colorado State University, CO, USA) under the TB research materials contract NIH, NIAID N01-A140091. Recombinants cloned in pET28a were grown in Luria–Bertani agar containing kanamycin 100 mg/L, ampicillin 100 mg/L and chloramphenicol 25 mg/L. The supernatant was loaded on an Ni2+-nitroacetic acid (NTA)–agarose (Qiagen, Hilden, Germany) column equilibrated with 50 mM sodium phosphate, pH 7.4, containing 0.5 M NaCl and Triton X-100 1% v/v. After washing the column with 100 mM imidazole in buffer A, bound Rv3872 was eluted with 250 mM imidazole in buffer A.

**Expression of recombinant antigens and purification**

His-Rv3872. *Escherichia coli* BL21(DE3)/Rv3872 was grown to OD600 0.6. Isopropyl-β-D-galactopyranoside (Sigma, St Louis, MO, USA) was added to a final concentration of 0.1 mM, and growth was continued at 37°C with shaking for 2 h. Cells were lysed with lysozyme at a final concentration of 1 mg/mL. The supernatant was loaded on an Ni2+-nitroacetic acid (NTA)–agarose (Qiagen, Hilden, Germany) column equilibrated with 50 mM sodium phosphate, pH 7.4, containing 0.5 M NaCl and Triton X-100 1% v/v (buffer A). After washing the column with 100 mM imidazole in buffer A, bound Rv3872 was eluted with 250 mM imidazole in buffer A.

His-ESAT-6 and His-CFP-10. *E. coli* BL21(DE3)/ESAT-6 or *E. coli* BL21(DE3)/CFP-10 was expressed by induction with 0.1 mM isopropyl-β-D-galactopyranoside at 37°C for 4 h. The cell pellets were lysed with Bugbuster HT (EMD Biosciences, San Diego, CA, USA), to which was added 0.5 mM EDTA and 100 µM phenylmethylsulphonylfluoride. ESAT-6 or CFP-10 was purified from the cell-free supernatant by chromatography on Ni2+-NTA-agarose, as described above. After washing the column with 50 mM imidazole in buffer A, ESAT-6 was eluted with 150 mM imidazole in buffer A.

GST-Rv3878. *E. coli* BL21/Rv3878 was expressed by induction with 0.1 mM isopropyl-β-D-galactopyranoside for 2 h at 37°C. Cells were lysed with lysozyme, with Rv3878 being purified from the cell-free supernatant by chromatography on glutathione (GST)–Sepharose (GE Healthcare Bio-Sciences, Kawi Chung, Hong Kong).

**Western blotting**

Recombinant antigens (20 µg) were separated by SDS-PAGE and transferred to nitrocellulose membranes (Millipore India, Bangalore, India). Each membrane was cut into strips and incubated for 2 h with sera from TB patients or healthy BCG-vaccinated individuals, diluted 1:200 in phosphate-buffered saline (PBS) containing Tween-20 0.3% v/v and bovine serum albumin 0.1% w/v (PBSTA). After washing, strips were incubated for 1 h at room temperature with protein A-peroxidase (BD Biosciences, San Diego, CA, USA) diluted 1:2000 in PBSTA. Detection was by means of 3,3′-diaminobenzidine (DakoCytomation, Glostrup, Denmark) and incubating with peroxidase-conjugated goat anti-human IgG (H+L) (DakoCytomation).

**Table 1. Primers and vectors used for cloning of RD1-encoded antigens**

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Sense primer * (5′ → 3′)</th>
<th>Antisense primer * (5′ → 3′)</th>
<th>Cloned between</th>
<th>Vector</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rv3872</td>
<td>ATGGGATCCATGAAAAAAATGCTACAT</td>
<td>TAGAATCTCTTCATTCCGGCGGAGACGCC</td>
<td>BamHI and EcoRI</td>
<td>pET28a</td>
</tr>
<tr>
<td>Rv3873</td>
<td>TTAGGATCCATGCCCCAGCCAGAAGTG</td>
<td>TAGAATCTCTTCATTCCGGCGGAGACGCC</td>
<td>BamHI and EcoRI</td>
<td>pET28a</td>
</tr>
<tr>
<td>Rv3874 (CFP-10)</td>
<td>TTGGATCCATGCTGTGGCACGCAATGTAT</td>
<td>TAGAATCTCTTCATTCCGGCGGAGACGCC</td>
<td>BamHI and EcoRI</td>
<td>pET28a</td>
</tr>
<tr>
<td>Rv3878</td>
<td>ATGGGATCCATGAAAAAAATGCTACAT</td>
<td>TAGAATCTCTTCATTCCGGCGGAGACGCC</td>
<td>BamHI and EcoRI</td>
<td>pET28a</td>
</tr>
</tbody>
</table>

*Restriction sites are underlined.

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of chemiluminiscence with Lumiglo reagent (Cell Signaling Technology, Beverly, MA, USA) and exposure to X-ray film.

**Synthetic peptides**

Eleven 20-mer peptides, overlapping by 12 amino-acids and covering the entire Rv3872 amino-acid sequence, were synthesised using solid-phase pin technology (Mimotopes, Clayton, Victoria, Australia). All peptides were dissolved in sterile distilled water at a concentration of 2 mg/mL and were stored frozen at −70°C until use. Stock peptide solutions were diluted to obtain the desired concentration in PBS.

**Antibody detection by ELISA**

Microtitre plates were coated with recombinant antigens (200 ng/well), blocked with PBS containing BSA 1% w/v for 2 h at room temperature, and then washed in PBS containing Tween-20 0.1% v/v (PBST). Serum (diluted 1:500) was added to the wells, and the plates were incubated for 1 h at room temperature, washed with PBST and incubated with anti-human IgG Fab’2 peroxidase (Sigma) (1:5000 dilution) for 1 h at room temperature. Horseradish peroxidase activity was detected by adding the chromogenic substrate o-phenylenediamine tetrahydrochloride (Sigma) and H₂O₂. The reaction was stopped using 1 M H₂SO₄ and readings were taken at 490 nm. Each ELISA was repeated on at least three occasions, with replicates for each sample within individual ELISAs.

**Data analysis**

Student’s t-test and ANOVA were used for statistical analyses. Sensitivity was defined as the ability to detect cases of active TB. Specificity was defined as the ability to yield negative results for the control groups, which were considered to be free of active TB. For evaluation of antibody responses, cut-off values were calculated for each antigen as the means of OD₄₉₀ values plus three SDs obtained with the sera from 123 healthy BCG-vaccinated donors.

**RESULTS**

**Expression and purification of antigens**

Rv3872, CFP-10 (Rv3874) and ESAT-6 (Rv3875) were all expressed as 6x His-tagged proteins in *E. coli* and were purified from cell-free supernatants by chromatography on Ni²⁺–NTA–agarose. Rv3878 was expressed as a GST-fusion protein and was purified by chromatography on GST–Sepharose. The purified proteins were analysed by SDS-PAGE and were detected by probing with anti-His or anti-GST antibodies (data not shown). Expression and purification results for Rv3872, Rv3878, CFP-10 and ESAT-6 are shown in Fig. 1.

![Fig. 1. Expression and purification of *Mycobacterium tuberculosis* proteins Rv3872, Rv3878, ESAT-6 and CFP-10. Coomassie blue-stained SDS gels showing (a) uninduced, (b) induced and (c) purified proteins. Protein marker sizes are indicated on the left side of each gel.](image)

**Western blot analysis of reactivity of recombinant proteins against sera from healthy BCG-vaccinated controls and TB patients**

Western blot analysis revealed that recombinant (r) Rv3872, ESAT-6, CFP-10 and Rv3878 reacted positively with sera from TB patients, but not with sera from healthy BCG-vaccinated individuals (representative blots are shown in Fig. 2).

**Recognition of RD1-encoded antigens by sera from healthy and infected subjects**

Sera from patients and healthy BCG-vaccinated individuals were tested against r-Rv3872, r-ESAT-6,
The sera were tested simultaneously against tuberculin (purified protein derivative; PPD). The data shown in Table 2 demonstrate that sera from all infected patients mounted a statistically significant (p < 0.01 — <0.0001) antibody response against r-Rv3872, r-ESAT-6, r-CFP-10 and r-Rv3878 when compared with that of healthy BCG-vaccinated individuals. However, sera from TB-infected patients did not show statistically significant differences in response to PPD when compared with sera from healthy BCG-vaccinated individuals. When the immunogenicity of Rv3872 was compared with that of ESAT-6 and CFP-10, the mean values for Rv3872 with pulmonary and extra-pulmonary TB were 1.32 and 0.62, respectively, compared with 0.39 and 0.29, respectively, for ESAT-6, and 0.35 and 0.28, respectively, for CFP-10. The mean values for Rv3878 with pulmonary and extra-pulmonary TB were 1.19 and 0.68, respectively (Table 2). Thus Rv3872 and Rv3878 elicited stronger immunoreactivity than ESAT-6 and CFP-10, and could discriminate patients with new pulmonary TB infections from healthy BCG-vaccinated donors (p < 0.0001 and p < 0.001, respectively, as tested by ANOVA, for the two proteins). These values suggested that Rv3872 is a better candidate for a diagnostic tool than Rv3878 in terms of distinguishing patients with pulmonary TB from healthy BCG-vaccinated individuals.

To further analyse the efficacy of each of the antigens tested, the number of sera reactive against Rv3872 and Rv3878 were counted (i.e., those sera with antibody levels greater than or equal to the mean OD490 of negative control sera plus 3 SD). The majority (>90%) of the responders showed levels of antibody to these antigens greater than the above cut-off values (Fig. 3).

**Sensitivity and specificity of antigens**

With present diagnostic methods, diagnosis of extra-pulmonary TB is more difficult than that of pulmonary TB. It would therefore be an advantage if a diagnostic tool capable of identifying this group of patients could be developed. In this context, Rv3872 and Rv3878 could discriminate extra-pulmonary TB patients from healthy BCG-vaccinated individuals with p values of <0.001 and <0.01, respectively (Table 2). For Rv3872, the sensitivities of detection of pulmonary TB and extra-pulmonary TB were 92% and 89%, respectively. For Rv3878, the values were 88% and 84% for pulmonary and extra-pulmonary TB, respectively. For Rv3872 and Rv3878, the specificities were 95% and 91%, respectively. Taken together, these results suggest that Rv3872 is a better candidate than Rv3878 for diagnosis of pulmonary and extra-pulmonary TB infections.

**Immunoreactivity against synthetic peptides covering the Rv3872 sequence**

Rv3872 was chosen in preference to Rv3878 for a detailed investigation into the potential serodiagnostic use of immunogenic peptides derived from its sequence. Overlapping peptides (P1–P11, Table 4) covering the entire Rv3872 sequence were synthesised in order to identify B-cell epitopes. These peptides were used to test for reactivity against the sera of patients with both categories of TB described above. Two overlapping peptides (P8 and P9) were identified as important antigenic epitopes of Rv3872. These peptides reacted strongly with the sera obtained from both categories of patients (Fig. 4). There was a four- to five-fold increase in the mean values, compared with normal individuals, when P8 or P9 were used individually with pulmonary TB patients (Fig. 4). With use of the mixture of P8 and P9, the mean value increased almost eightfold (p <0.0001) (Table 2).

The pooled P8 and P9 peptides, like the full-length protein, showed a significantly higher response against pulmonary TB infections than against extra-pulmonary TB infections (Fig. 4, except...
The specificity of the peptide cocktail was 100% and the sensitivities were 94% and 90%, respectively, for pulmonary and extra-pulmonary TB (Table 3). As with the full-length protein, <10% of sera from BCG-vaccinated healthy individuals showed an antibody response above the cut-off value (Fig. 3). These peptides showed a stronger immunoreactivity for patients with pulmonary TB infections than for BCG-vaccinated healthy individuals (Fig. 4) (p < 0.0001).

Rv3872 was scanned to identify regions of high antigenic index using the online antigenic peptide prediction software available at http://immunax.dfci.harvard.edu, following the method of Kolaskar and Tougaonkar [7]. Three antigenic sites, encompassing amino-acids 21–52, 57–65 and 68–85, respectively, were predicted. Peptides P8
and P9 encompass amino-acid residues 57–76 and 65–84, respectively, thereby explaining the observation that these peptides of high predicted antigenicity elicit strong immunoreactivity when tested by ELISA.

**DISCUSSION**

Considering that India accounts for one-third of the global TB burden, early cost-effective diagnosis is a priority. The present study evaluated the serological response to a panel of recombinant RD1-encoded antigens of *M. tuberculosis*. PPD failed to discriminate TB patients from healthy BCG-vaccinated individuals, thereby corroborating earlier observations [8,9]. The promise of an antigen as a serodiagnostic tool depends not only on the number of antibody responders among TB patients, but also on the level of response obtained with an antigen. Compared with CFP-10 and ESAT-6, the differences in mean absorbance values among TB patients and healthy BCG-vaccinated individuals were much higher for Rv3872 and Rv3878 (Table 2). However, Rv3873 gave a positive reaction with sera from healthy BCG-vaccinated individuals as well as with sera from TB-infected patients (unpublished results), and was therefore excluded from further study.

The present findings contradict those of earlier workers [10], who reported that only 3–7% of TB patients responded to RD1 region proteins, including Rv3872 and Rv3878, although all RD1 antigens elicited a high antibody response in guinea-pigs infected with *M. tuberculosis*. This is not surprising, since the serological response to a

<table>
<thead>
<tr>
<th>Peptide no.</th>
<th>Sequence</th>
</tr>
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<tbody>
<tr>
<td>P1 (1–20)</td>
<td>MEKMSHPDAADIGTQVSDSN</td>
</tr>
<tr>
<td>P2 (9–28)</td>
<td>IAADIGTQVSDNALHGVTAG</td>
</tr>
<tr>
<td>P3 (17–36)</td>
<td>VSDNALHGVTAGSTAISVT</td>
</tr>
<tr>
<td>P4 (25–44)</td>
<td>VTAGSTALTSYTGLVFAGAD</td>
</tr>
<tr>
<td>P5 (33–52)</td>
<td>TSYTGLVFAGADEVSAQAAT</td>
</tr>
<tr>
<td>P6 (41–60)</td>
<td>ACADEVSQAATAFPSGQ</td>
</tr>
<tr>
<td>P7 (49–68)</td>
<td>QAAFTSEGQILNASANA</td>
</tr>
<tr>
<td>P8 (57–76)</td>
<td>EIGQLNASNASDQQLRAG</td>
</tr>
<tr>
<td>P9 (65–84)</td>
<td>NASDQQLRAGAEVQDOVAR</td>
</tr>
<tr>
<td>P10 (73–92)</td>
<td>HRAGEAVQDVARTYSQIDDG</td>
</tr>
<tr>
<td>P11 (81–99)</td>
<td>DVAARTYSGIDGAAGVFAE</td>
</tr>
</tbody>
</table>

Table 4. Amino-acid sequences of synthetic peptides derived from the Rv3872 sequence

and P9 encompass amino-acid residues 57–76 and 65–84, respectively, thereby explaining the observation that these peptides of high predicted antigenicity elicit strong immunoreactivity when tested by ELISA.
specific antigen often depends on the geographical location and ethnic background of the population being studied. For example, large variations in sensitivity have been reported using the same antigens against sera from TB patients living in Denmark and those living in Uganda [11]. In addition, Weldingh et al. [11] have suggested that the magnitude of the antibody response depends on the stage of disease at which patients from areas of high and low endemicity are admitted to hospital.

Genetic as well as environmental factors also influence the antibody response. The diverse antibody response to \textit{M. tuberculosis} is governed by HLA types. Arend et al. [12] reported that interferon-\(\gamma\) production by peripheral blood mononuclear cells of TB patients was significantly higher in the presence of HLA-DR15, which is a major subtype of DR2. It is therefore not surprising that, contrary to the results of studies in western countries, Rv3872 appears to be a promising antigen for developing serological diagnosis kits for detecting TB in the context of an Indian population residing in an endemic area.

In conclusion, the data presented in this study highlight the potential of the recombinant RD1-encoded antigen Rv3872, as well as the peptide cocktail derived from this protein, for serodiagnosis of both pulmonary and extra-pulmonary TB. The promising results obtained with the peptide cocktail argue in favour of its inclusion in serodiagnostic ELISA kits for detecting TB. Additional efforts are now being made to develop serological approaches for detection of smear-negative infections using the peptides derived from Rv3872. The practical utility of using such a peptide cocktail for differentiating fresh infections from relapsed infections is an additional aspect that deserves further investigation.

**ACKNOWLEDGEMENTS**

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**REFERENCES**