Humoral immune responses of normals and tuberculosis patients to multiple sonicate antigens prepared from the most prevalent strains of *Mycobacterium tuberculosis* harbouring single copy of IS6110 from South India

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The search for newer species-specific antigens from prevalent strains of *Mycobacterium tuberculosis* (Mtb) associated with active disease will add substantially to improve the presently available diagnostic tools. We studied the protein profiles of 13 sonicate antigens (S1– S13) prepared from the prevalent strains. The humoral immune response was also studied in 30 normal and 30 tuberculosis (TB) patients. The sonicate antigens S10 and S12 showed maximum differential protein bands in low molecular mass region of 10–30 kDa on SDS–PAGE. Our ELISA results showed significant increase in Mtb-specific IgG antibodies in TB plasma for H37Rv, followed by PPD, S1 and S10 antigens. Immunoblot analysis of S10 antigen showed specific recognition pattern at low molecular mass region by TB plasma alone with 12–77% positivity of protein bands. Thus the sonicate antigen S10 was found to be discriminatory by ELISA and Western blot and hence a good candidate for further purification of its individual proteins to be evaluated for diagnosis.

Keywords: Humoral immune response, prevalent strains, *Mycobacterium tuberculosis*, sonicate antigens.

FOR several decades, rigorous investigations are being performed to understand the humoral immune response to infection by *Mycobacterium tuberculosis* (Mtb). The primary purpose of these investigations was to devise specific and sensitive serodiagnosis for tuberculosis (TB)[1–6]. Many studies have shown the diagnostic values of native[7,8] and recombinant[9–11] antigens of Mtb, but single antigen-based assays could not achieve satisfactory serodiagnostic performance[12,13]. Moreover, these studies have used antigenic components from the laboratory strains of Mtb (H37Rv, H37Ra) and non-human strains (*M. bovis*, *M. bovis* BCG) for evaluation. Most of the non-specificity observed in these assays was due to their widely shared antigens among species and genera. In addition, the relatively poor immunogenicity of many of the specific and purified antigens seemed to be another limitation[14].

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Recently, identification of the RD1 region in the Mtb genome that is absent in BCG and most non-tuberculous mycobacteria provided an opportunity to develop new specific diagnostic tools by rational design[15–17]. But this approach has other complications, which have to be managed efficiently[1]. However, in one study, the RD1 region encoded antigens ESAT-6 and CFP-10 and their peptides and epitopes were used to study the latency of Mtb infection in India[17].

At present, efforts are directed towards the identification of newer antigens that are associated with active disease. This type of approach will lead to the development of improved strategies for immunodiagnostics of TB. Thus the major objective of current immunological studies in TB is the identification of species-specific antigens and determination of the significance of corresponding immune responses.

In this study, we prepared the sonicate antigens from recent and prevalent strains of Mtb which had a single copy of insertion element IS6110 and showed 40% of prevalence based on our molecular epidemiological studies[18,19]. We analysed their protein profiles using SDS– PAGE and compared their humoral immune response with the standard strain H37Rv in normal and TB patients by ELISA and Western blot.

Totally 13 clinical strains (S1 to S13) obtained from BCG trial area of Tiruvallur district, Tamil Nadu formed the major clusters by IS6110 and other secondary typing methods[19,20]. Of the total strains infecting in South India, the less prevalent (1–2%) strains (S1 and S2, S3) had IS6110 insertion at 4.5 kb and 1.0 kb respectively, while the most prevalent (38%) strains (S4–S13) had the insertion in hotspot region of Direct Repeat (DR) locus at 1.5 kb. These clinical isolates were first grown on Lowenstein–Jensen's slopes and then transferred and grown in Sauton's broth medium (without Tween 80) for preparing sonicate antigens. The cultures were grown stationary as surface pellicle. The sonicate antigens were prepared from these strains by sonicating the bacilli in Soniprep 150 as described earlier[21]. The same lot of each antigen was used throughout the study to avoid variations. These antigens and H37Rv were resolved on 10% SDS–PAGE[22] and the protein profiles were analysed and compared using the software Bio-Capture (Labmate, USA).

The blood plasma was collected from 30 PPD-positive healthy laboratory volunteers (Nor) and 30 active pulmonary TB patients with age ranging from 22 to 60. All the patients were sputum smear-positive and were seronegative for HIV. Written informed consent was obtained from each patient and the study followed the ethical guidelines of the Government General Hospital, Chennai.

Antibody titre of normal and TB plasma against these antigens was determined by ELISA using 5 μg/ml sonicate antigen concentration for coating and 1 : 50 dilution of plasma which gave the optimum reproducible results in a linear range. The end colour was read at 450 nm in

ELISA reader molecular devices, SPECTRA MAX-250, USA.

Immunoblot was performed by transferring electrophoretically resolved proteins onto polyvinylidene difluoride membrane (Bio-Rad, USA) and further tested with 1 : 100 dilution of ten normal and ten tuberculous plasma. The molecular weight was calculated with reference to pre-stained marker (GIBCO, USA). The immunoblots were further analysed based on actual molecular weight and percentage positivity of each band. Arithmetic mean and cut-off values were calculated for antibody ELISA. The significance of differences was estimated by Student's paired t-test.

Several attempts have been made to purify specific antigens from Mtb for use as diagnostic reagents[23]. These purified antigens and their epitopes gave better specificity but could not improve the sensitivity[24]. To improve the sensitivity of the diagnostic test, we need a crude and/or complex preparation of antigens with minimum cross-reactivity. Such antigenic preparations can be obtained by testing newer antigenic preparations from the recent Mtb strains, which are actively involved in infection and transmission of the disease. This model approach can then be applied to identify more antigens from other strain pools.

Here, we analysed the protein profiles of these sonicate antigens and evaluated their diagnostic potential. The protein profiles showed maximum differences in low molecular mass region of 10–30 kDa, more distinct in S10 and S12 antigens (Figure 1). Many studies have shown the differential expression of proteins which could be growth stage-specific, or associated with virulence factors when

Figure 1. Protein profiles of sonicate antigens (S1–S13) of prevalent *Mycobacterium tuberculosis* strains from clinical isolates. Lane 1, Protein profile of H37Rv sonicate antigen; lanes 2–14, Sonicate antigens from Mtb strains; lane 15, Molecular weight marker. Some differential and upregulated protein bands are marked with arrows.

expressed under stress conditions[25]. As Mtb strains in this study were cultured and harvested under the same culture conditions, the observed differences in protein profiles might be truly differential and may not be directly related to virulent factors or growth stage-specific expression. Also many studies have evaluated the proteins released during growth and shown the importance of low molecular weight proteins in immunogenicity and immunodiagnostics[26–30]. Our observations of differential proteins in S10 and S12 may prove important in the search of new antigens from these preparations.

Antigens of Mtb should be immunogenic and trigger appropriate antibody response in infected and TB patients[31]. We studied the antibody response of S1, S2, S10 and S12 antigens to assess their usefulness in diagnosis. As expected, significantly high ($P < 0.0001$) antibody titre was obtained in TB plasma against H37Rv, PPD, S1 and S10 antigens compared to normals (Figure 2). The difference was also greater and significant in pooled TB plasma compared to pool normal. The number of patients showing higher antibody titre than the cut-off value (mean + 2 SD) was 19/30 in H37Rv, 18/30 in S1, 15/30 in PPD and 13/30 in S10. Only one healthy individual showed antibody levels above cut-off value and hence the false positivity was only 3%.

Thus the sonicate antigens S1 and S10 showed significant increase in antibody levels in TB plasma and were comparable with H37Rv, indicating the presence of potential immunogenic antigens in these clinical strains. It is also well established that antibody response during TB varies extensively and no single antigen can adequately cover such diverse antibody repertoire and therefore yield an accurate serodiagnostic assay. To overcome this, recently, a multi-antigen serological approach had been used for diagnosis of TB[32,33]. The antigen preparation S10, if dissected further by 2D gel electrophoresis and purified into individual new proteins, may prove more useful in multi-antigen serological approach.

Since these are prevalent strains, we are bound to pick higher responses to them within the community and in patients. Therefore, higher antibody response alone may not provide sufficient information regarding the specificity of newer epitopes present in the antigens. Hence, these quantitative differences were further substantiated with the more qualitative data of Western blot.

With the differential protein profiles and promising antibody response by ELISA, antigens S1 and S10 were further tested using Western blot to decipher if the protein bands of differential intensity in PAGE were exclusively recognized by TB antibodies to prove their immunodominance. Surprisingly, in our immunoblot results, S10 alone showed specific recognition pattern in low molecular weight regions by only TB plasma showing absolute specificity (Figure 3). Immunoblots of H37Rv and S10 were further compared and percentage positivity for each band was calculated. For S10 antigen, most of the bands were recog-
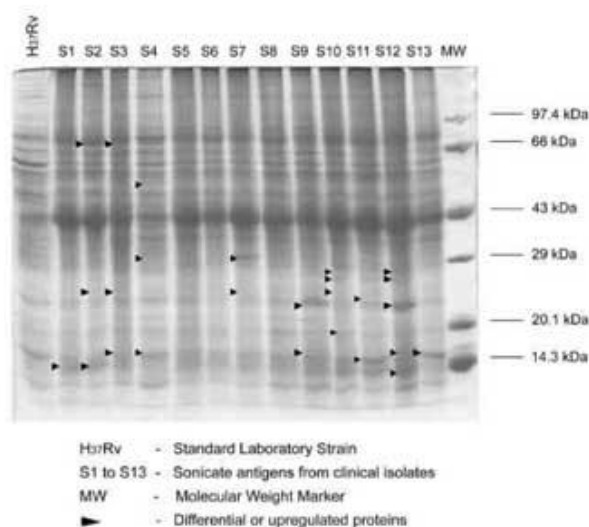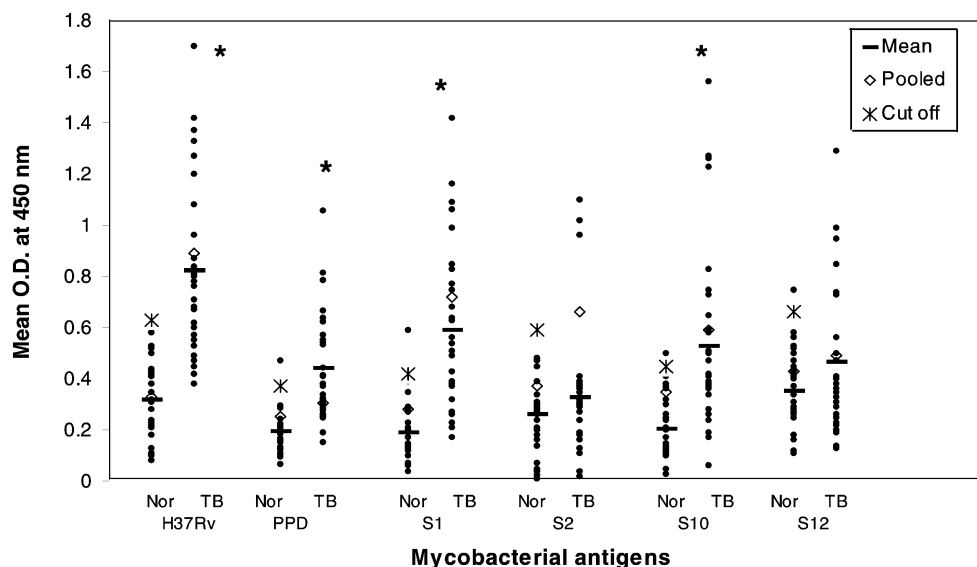
Figure 2. Total IgG antibody levels by ELISA in 30 normal and 30 pulmonary TB patients against various mycobacterial sonicate antigens and comparison with standard H37Rv and PPD. Statistical significance is shown as *$P < 0.0001$.
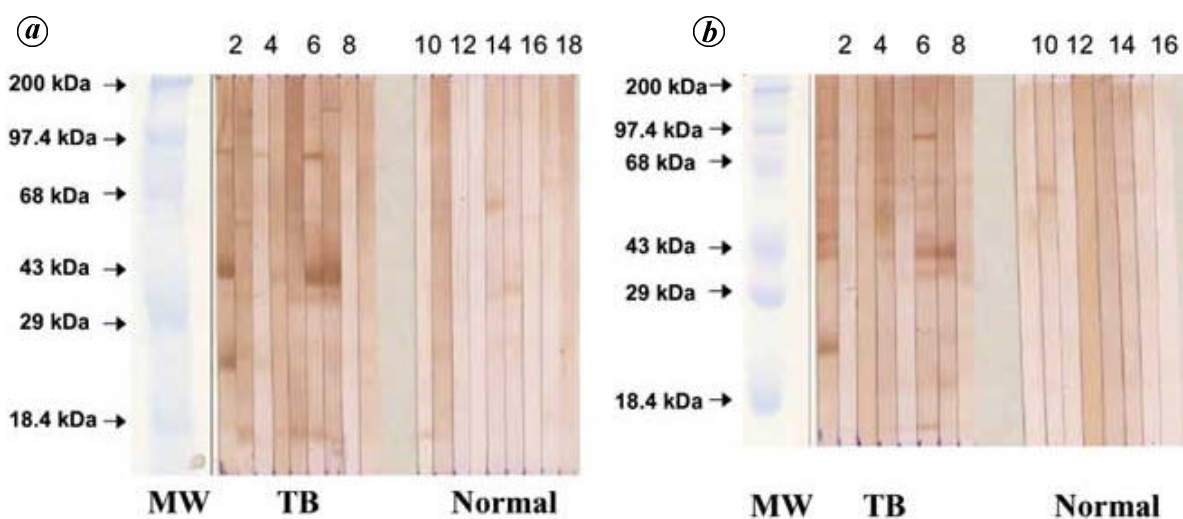

Figure 3. Representative immunoblot analysis for H37Rv (*a*) and S10 (*b*) antigens with 9 and 8 subjects respectively in both TB and normal groups. Prestained molecular weight marker is shown in lane 1.

nized only by TB plasma with 12–77% positivity. Maximum positivity was observed for 16 and 45 kDa (77%) followed by 38 kDa (66%) protein bands (Figure 4). We speculate that such specifically recognized antigens in the Western blot of S10 might have contributed to higher antibody titre in S10 ELISA results. Many studies have reported varied antibody response to various antigens of Mtb by Western blot[34]. Recently, new antigens from culture filtrate proteins of Mtb were identified for differentiating

HIV-positive individuals who are at high risk for developing TB[35].

The criteria for selection of one antigen preparation out of 13 for further purification of immunogenic proteins were based on the differential protein profile and antibody response. Although we observed that S2 and S12 showed many differential proteins on gel, their discriminatory power in antibody ELISA was not good. On the other hand, antigen S1 showed few differential bands on SDS–
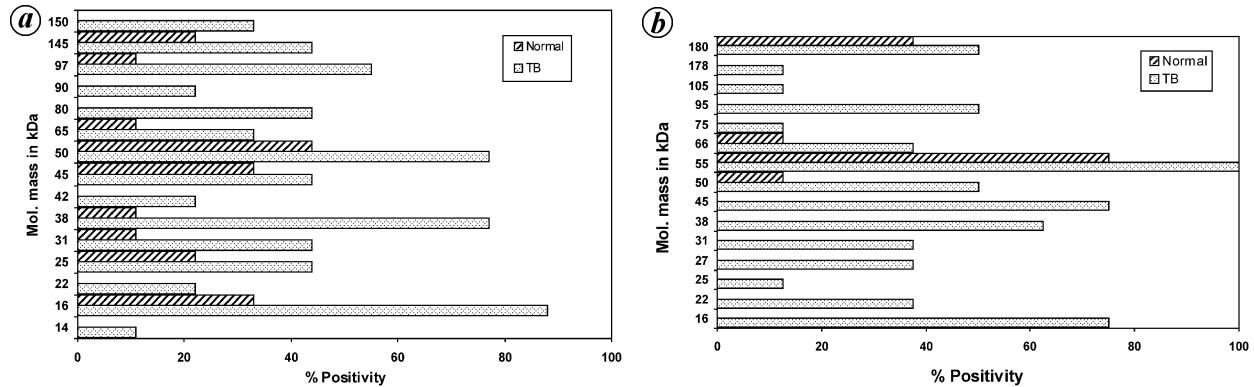
Figure 4. Percentage positivity of various protein bands in 10 normals and 10 TB plasmas for H37Rv (*a*) and S10 (*b*) antigens was calculated and presented graphically. The recognition of each protein band by the number of TB and/or normals was converted into % (percentage positivity). Percentage positivity of both TB and normals was drawn against each protein band.

PAGE but its performance for antibody ELISA was discriminatory, better than PPD and comparable to that of H37Rv. Though the protein profiles observed on SDS–PAGE need not reflect in the antibody profiles on ELISA or blot, this observation indicates the complexity of the protein profiles and their differential response. Moreover, the culture of Mtb clinical isolates and H37Rv in stress conditions expresses certain proteins, which can be attributed to virulence-associated proteins[25]. Thus it was difficult to select the more potent and more discriminatory antigen preparation based on the above criteria from these widely spread strains in the community.

In our earlier study, we found that these sonicate antigens induced differential cell-mediated immune responses in healthy laboratory volunteers[21]. Here we further show that these antigens have the potential to induce differential humoral response. The diverse properties of these antigens emphasize their importance in evaluating the diagnostic potential, virulence associated factors and various immune parameters. In the recent past several studies were conducted with similar views using many outbreak strains[36–40]. In one such study[38] it was shown that clinical strain CDC1551 elicited a more vigorous host immune response than H37Rv. Analysis of the proteome identified several quantitative differences in the cellular protein composition of these strains[39]. A similar type of study was conducted with another outbreak strain belonging to the Beijing family and a comparison was made for its protein profile and antigen recognition with H37Rv and a less prevalent clinical strain. The result of this study showed differences in the protein profile which were further attributed to the outbreak strain's successful spread in the community[40].

We made use of these basic and preliminary immunological methods to screen more antigenic preparations and found gross differences, which can be used for tapering the search for one or two antigens. By this approach we could identify some differences in SDS–PAGE protein profiles for antigens S10 and S12, and strong antibody response to S1 (ELISA) and S10 (ELISA and Western blot). Although the study uses limited methodology, it invokes convincing scope for further evaluation of these strains using state-of-the-art technology in the field of diagnostic research. Now we are confining to antigen S10 for further studies on purification and characterization of individual proteins either by conventional procedures or by proteomics approach.

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