

## EVALUATION OF *MYCOBACTERIUM TUBERCULOSIS* ANTIGEN 6 BY ENZYME LINKED IMMUNOSORBENT ASSAY (ELISA)

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**Summary.** *Mycobacterium tuberculosis* Antigen 6, which is a 30 KDa protein purified by DEAE-Cellulose chromatography, was evaluated by enzyme linked immunosorbent assay (ELISA) for its sensitivity and specificity for antibody detection in sera from patients with pulmonary tuberculosis. Purified Protein Derivative (PPD) was used for comparison. Antigen 6 could detect 89% of bacteriologically confirmed cases and 32% of bacteriologically negative cases. However, 25% of the healthy volunteers also had positive antibody levels. Among the treated, inactive cases of tuberculosis 44% had higher antibodies. Thus, because of the low specificity and persisting antibody titres, Antigen 6 cannot be used in diagnostic or prognostic tests.

### Introduction

The need for highly sensitive and specific serodiagnostic tests for tuberculosis is well recognised. The limitations associated with the bacteriological confirmatory tests, such as lower sensitivity of the smear and time-consuming nature of the culture, make it imperative that alternative tests be developed. Since the first description of Enzyme Linked Immuno-Sorbent Assay (ELISA) for tuberculosis by Nassau *et al*<sup>1</sup>, the assay has been widely tried in serodiagnosis, using crude extracts as well as purified fractions. Numerous groups of workers in the past have used various physico-chemical methods, such as serial ammonium sulphate precipitation, molecular exclusion ion-exchange, chromatography, acrylamide gel

electrophoresis etc., for purification of antigen<sup>2,5</sup>. Recently Nagai *et al*<sup>6</sup> have described a serial chromatography procedure for the isolation of major antigens of *Mycobacterium tuberculosis*.

One of the antigens of *M. tuberculosis*, which is predominantly present as a secretory protein in the culture filtrate, namely Antigen 6, has been purified using DEAE Cellulose chromatography<sup>7</sup>. Antigen 6 was characterized and described as a 30 KDa protein. It has been evaluated for its diagnostic potential in a study population in Argentina<sup>8</sup> and found to be useful. In the present study, we have evaluated the performance of *M. tuberculosis* Antigen 6 by ELISA for the serodiagnosis of pulmonary tuberculosis patients from Madras, south India.

### Material and Methods

#### Sera

Sera were collected from tuberculosis patients and normal healthy individuals. The study groups are described in Table 1. All sera were stored at -20°C until use.

In all, 118 sera were included in this study, of which 62 were obtained from tuberculosis cases, 29 from normal healthy volunteers (employees of Tuberculosis Research Centre- TRC), 11 from cases with non-tuberculous lung disease like asthma, allergy etc., and 16 from cases with inactive pulmonary tuberculosis. The sera from tuberculosis cases could be divided into (i) bacteriologically confirmed cases (N = 45) (S+ C+) and (ii) bacteriologically negative, but

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clinically and radiologically diagnosed cases (N = 17) (S- C-). In the inactive tuberculosis group, all the 16 subjects were positive by smear and culture before chemotherapy, but became quiescent at the end of therapy.

For bacteriological testing, 4 sputum specimens were obtained from each individual (2 spot specimens and 2 overnight collections). Two smears were made from each of the specimens, stained with auramine-phenol and counted under the fluorescent microscope. The smear gradings were done as described previously<sup>9</sup>. For culture, each sputum was treated by Petroff's method and inoculated on 2 L-J slopes. The growth was followed for 8 weeks before classifying the sputum as negative. The culture grading was done as previously described<sup>10</sup>.

#### Antigens

The antigens used in the assay are Purified Protein Derivative (PPD) (Central Veterinary Laboratory, Weighbridge, England) and *M. tuberculosis* Antigen 6, which was given as a kind gift by Prof. Thomas Daniel, Case Western Reserve University, Cleveland, USA.

It is a 30 KDa protein purified from unheated culture filtrates of H<sub>37</sub>Ra by DEAE-Cellulose chromatography.

#### Enzyme Linked Immunosorbent Assay (ELISA)

Polyvinyl microtitre plates (96 wells, U bottom, Flow Laboratories, USA) were coated with 100 µl of PPD or Antigen 6 (5 µg/ml) per well. For coating, the antigens were diluted in 0.06 M Sodium carbonate-bicarbonate buffer, pH 9.6 and incubated overnight at 4°C. The plates were washed thrice with PBS containing 0.1% Tween 20. In order to block the non specific binding sites, the wells were incubated with 1% bovine serum albumin (BSA) for 1 hour at 37°C. After 3 washings the plates were incubated with 1 : 100 dilution of the sera from patients and normals. The plates were washed after 1 hour of incubation at 37°C and antihuman IgG-Peroxidase (1/1000, Sigma) was added as the second antibody. At the end of 1 hour of incubation at 37°C and washing, the colour was developed by the addition of 100 µl of the substrate (30 mg/50 ml of O-Phenylene

**Table 1.** Description of study population

Subjects	No. of specimens	Category
Pulmonary TB patients positive by smear and culture	45	S+ C+
Pulmonary TB patients negative by smear and culture	17	S- C-
Healthy laboratory staff of T.R.C.	29	NHS*
Patients with asthma and allergy (non-tuberculous diseases) from General Hospital, Madras	11	NHS*
@ Pulmonary TB patients one and half years after stopping successful chemotherapy	16	in active
<b>Total</b>	<b>118</b>	

@ All these were positive by smear and culture (S+ C+) before start of chemotherapy and they became quiescent at the end of chemotherapy

\* Non-TB/Healthy Subjects

diamine and 30  $\mu$ l of 30%  $H_2O_2$ . After arresting the reaction with 50  $\mu$ l of 8N  $H_2SO_4$ , the optical density (O.D.) was read at 490 nm in Biotec Microplate, Autoreader EL290 (Biotec Instruments Inc., USA).

While each specimen was set up in duplicate in each plate, the cut-off point standard was set up in quadruplicate (from previous ELISA results, 5 normal sera which were consistently giving O.D. ranging from mean to mean + 2 S.D. of the normal sera were pooled for the reference standards). A test specimen was classified as positive if its mean O.D. was greater than or equal to the mean O.D. of the standard assayed in the same plate; otherwise, negative.

All the test specimens were coded before the assays were undertaken. Employing three plates for each antigen, assays for all the 118 specimens were completed in one day. On the second day, the assays were repeated under identical conditions.

#### Statistical analysis

Statistical analysis was done using

Statworks<sup>TM</sup> Version 1.1 (Cricket Software, Inc., PA, USA). Student's t-test was used to compare the group means. In addition, Multiway Anova and nonparametric McNemar test were also employed for comparison.

#### Results

The sera belonging to the 4 categories shown in Table 1 were tested in ELISA with PPD and Antigen 6. The results are shown in Fig. 1a and 1b. Each point represents the mean O.D. obtained with one individual serum. The group means as well as the cut-off point values (0.276 for PPD and 0.296 for Antigen 6) are marked.

In the S+ C+ group, the mean antibody levels against both PPD and Antigen 6 were significantly elevated ( $P < .0001$ ) as compared to NHS. In the S- C- group also, the group means were significantly elevated ( $P < 0.02$ ). Between the two groups of tuberculosis, S+ C+ had a higher mean O.D. for both the antigens, as compared to S- C-. The mean anti-PPD antibody levels were significantly

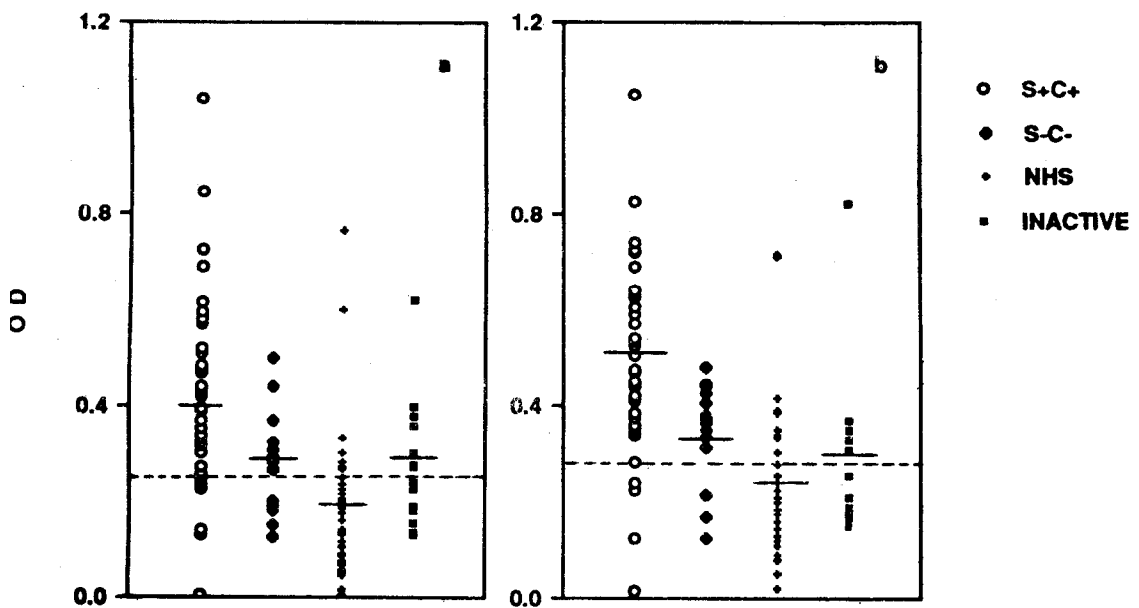


Fig. 1a. Antibody level against PPD in four groups as indicated in Fig. Each point represents the mean O.D. of an individual serum. Group means are indicated by solid horizontal lines; broken horizontal line indicates the cut-off point.

Fig. 1b. Antibody level against Antigen 6 in four groups as indicated in Fig. Each point represents the mean O.D. of an individual serum. Group means are indicated by solid horizontal lines; broken horizontal line indicates the cut-off point.

**Table 2.** Percentages of ELISA positive results among tuberculous patients (S+ C+ and S-C-)

Antigen	S+ C+		S- C-	
	No. positive	% positive	No. positive	% positive
PPD	31/45	69	9/17	53
Antigen 6	40/45	89	14/17	82

S+ C+ = Smear positive culture positive tuberculosis  
S- C- = Smear negative culture negative tuberculosis

**Table 3.** Percentages of ELISA negative results among healthy individuals and cured patients

Antigen	Healthy staff & asthma and allergy patients			"Cured" TB patients		
	No. of sera	No. negative	% negative	No. of sera	No. negative	% negative
PPD	40	35	88	16	8	50
Antigen 6	40	30	75	16	7	44

higher than normals ( $P < 0.02$ ) in the "Inactive" group, but the antibodies to Antigen 6 were not so.

The number and percentage of sera positive in S+ C+ and S- C- groups, for PPD and Antigen 6 are indicated in Table 2. The sensitivities of PPD and Antigen 6 were 69% and 89% respectively, in the S+C+ group, while the specificities were 88% and 75%. Antigen 6 worked better in the S-C- group, detecting 14 positives out of 17, while PPD detected only 9 out of 17. The number and percentage of sera negative in ELISA among the NHS and "Inactive" are shown in Table 3. The specificities were 88% and 75% with PPD

and Antigen 6.

Eight and 7 among the sixteen "Inactive" tuberculosis patients (18 months after completion of therapy), were positive for PPD and Antigen 6 respectively. Sera collected from 5 of these positive individuals, at various time periods during the treatment (0, 1, 6, /8, 12, 15, 18 and 24 months after start of treatment), were compared for their antibody levels. IgG antibodies against Antigen 6 were detectable at all time periods and found to be getting lower during the first month of treatment (the extent varies from patient to patient) (Fig. 2). Thereafter, the levels either increased by 6 months or remained steady for

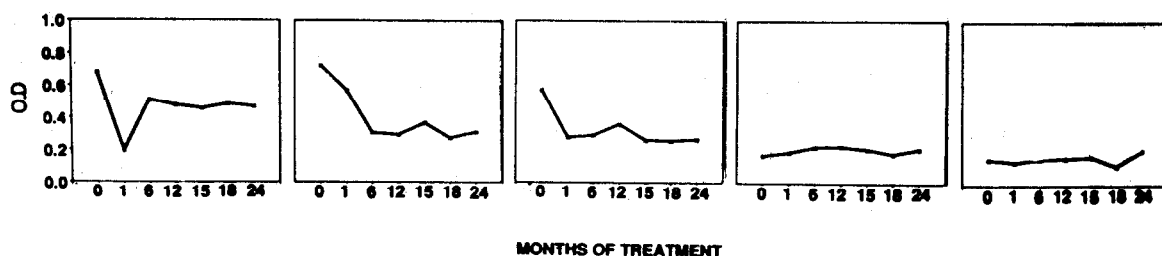


Fig. 2. Antibody level against Antigen 6 in sequential serum samples collected from 5 representative patients. The points denote the time of collection after the start of treatment : 1, 1, 6/8, 12, 15, 18 and 24 months.

as long as 24 months. Similar results were obtained for PPD also (Data not shown).

Table 4 shows the diagnostic test characteristics achieved by ELISA. Positive predictive values (S+C+ Group) for Antigen 6 and PPD were 0.80 and 0.86 respectively, while negative predictive values were 0.86 and 0.71. The positive predictive values were almost similar for both the antigens in both the groups (S+C+ and S-C-). However, Antigen 6 has a higher negative predictive value (0.86 as against 0.71). In the S-C- group also, the negative predictive value for Antigen 6 was higher than that of PPD (0.91 as against 0.81).

### Discussion

Antigen 6 used in this study is a well characterized antigen<sup>11</sup>. It is a purified, homogeneous 30 KDa protein of *M. tuberculosis*. Antigen 6 is one of the major secreted antigens of *M. tuberculosis*. It is a widely cross reacting antigen between different mycobacterial species. For example, Antigen 6 is identical to one of the antigens in BCG 86 complex, comprising 3 proteins of molecular weight 29-31KDa<sup>12</sup>. Isolation of this antigen is achieved by simple physicochemical procedures like ion exchange chromatography<sup>7</sup>.

The sensitivity of detection in the S+C+ group was 69% for PPD and 89% for Antigen 6, while specificity was 88% and 75% respectively. PPD classified 5/40 NHS as

positive, Antigen 6 classified 10/40 as positive. NHS group consisted of 2 sub-groups : TRC volunteers and those with non mycobacterial lung diseases. Out of the 11 non-mycobacterial lung disease patients, only one was identified as positive by Antigen 6. The additional 9 false positives happened to lie in the group of TRC volunteers. In the SC-group, 14/17 patients were identified as positive by Antigen 6, while only 9/17 were recognised as positive by PPD. Thus, Antigen 6 was more sensitive and more efficient in detecting disease at an earlier stage than PPD. This observation corroborates well with the in vitro observation<sup>13</sup> that large quantities of this antigen could be found in the culture supernatants as early as day 3. Since it is an early antigen, there is a likely hood of Antigen 6 being produced in large quantities at earlier stages of the disease process and stimulating antibody production before other bacteriological parameters become measurably high.

In the "Inactive" group, 8/16 and 7/16 sera remained positive for PPD and Antigen 6, respectively. For 5 of these patients, the test was repeated with sera collected at various time periods during therapy (0, 1, 6/8, 12, 15, 18, and 24 months after start of treatment). Antibodies to PPD and Antigen 6 were positive at all time periods. There was an initial lowering at 1 month (the extent varied from patient to patient) and the level increased by the 6th month or remained constant upto 24 months. During treatment,

**Table 4.** Diagnostic test characteristics (95% confidence limits) achieved using ELISA with PPD and Antigen 6

Antigen	Category	Sensitivity	Specificity	Predictive Values	
				Positive	Negative
PPD	S+C+	.69 (.53-.82)	-	.86 (.70-.95)	.71 (.57-.83)
	S-C-	.53 (.28-.77)	-	.64 (.35-.87)	.81 (.66-.92)
	NHS	-	.88 (.73-.96)	-	-
Antigen 6	S+C+	.89 (.76-.96)	-	.80 (.66-.90)	.86 (.73-.97)
	S-C-	.82 (.56-.96)	-	.58 (.37-.78)	.91 (.75-.98)
	NHS	-	.75 (.59-.87)	-	-

the antibodies did not correlate with the bacteriological status of the patients, since all these patients converted to sputum negativity within 1 month of treatment. Therefore, antibodies for Antigen 6 are not of much prognostic significance.

Persistence of antibodies long after the completion of therapy has been reported for other purified *M. tuberculosis* antigens like Antigen 5<sup>14</sup>. They persist for more than 24 months and in many patients upto several years. In a recent ELISA study with Antigen 6, Sada *et al*<sup>8</sup> have observed that 1 out of 6 inactive pulmonary tuberculosis patients had high antibody titres for Antigen 6.

The sparseness of specific antibody response before treatment is quite striking despite the general hyperglobulinaemia present in tuberculosis. One explanation offered is that the mycobacterial lipid coat insulates the cell wall glycoproteins and thereby prevents stimulation of humoral immunity. Antibiotic therapy results in the death of mycobacterial cells and release of antigen., which elicit a higher antibody response<sup>15</sup>. There is also a strong evidence for the action of suppressor cells in tuberculosis. The antibody response as measured by plaque forming cell assay (PFC) was drastically reduced in tuberculosis patients and found to increase after treatment<sup>16</sup>. Using the soluble antigen-fluorescent antibody test, Toussaint *et al*<sup>17</sup> have shown that pre-existing antibody levels increased reaching maximal levels within 60-90 days. Janicki and co-workers<sup>18</sup> have observed increased concentration of antibodies post-treatment, in the one dimensional immunoelectrophoresis technique. This effect was maximal after the third month of treatment. Similar findings were reported by Cole *et al*<sup>19</sup> also, by employing latex agglutination test. While all the above mentioned studies have used crude extracts, our study was carried out with a single, purified Antigen 6 and the enhancement of antibody synthesis after the treatment could still be observed.

In the present study, the specificity of Antigen 6 is not very high, even though the sensitivity is 89%. Therefore, ELISA using Antigen 6 by itself can not work as a good

diagnostic test. To evaluate its potential as a screening agent, it has been planned to collect a large number of finger prick blood specimens and subject them to ELISA test.

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