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Study of Immunoglobulin G subclasses to *M.tb.* Specific antigens in pulmonary tuberculosis patients with chemotherapy and their healthy house hold contact

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Abstract

Tuberculosis (TB) is a growing health problem in the developing world. Elimination of tuberculosis (TB) largely depends upon definitive rapid diagnosis and treatment. Widely used diagnostic tests do not qualify for use in a developing country due to lack of either desired accuracy or their cost. In the present study an enzyme-linked immunosorbent assay was used to evaluate the diagnostic potential of an immuno-dominant 30/32-kDa mycolyl transferase complex (Ag85 complex). Studies of antibody response in TB have focused mainly on their usefulness as a diagnostic serological tool, with little attention given to analysis of antibodies at the isotype and subclass level in relation to disease pathogenesis. Study speculate that chemotherapy kills bacteria leading to release of a great amount of cytosolic antigens increasing specific antibody levels to antigen 85 complex antigens after the first term of treatment. One explanation might be large load of bacteria. The answer this question was obtained by our follow up study of patients with chemotherapy, and the observation that the cure of the disease restores the response to against 85 complex. Furthermore, the higher level of these antibodies with high bacillary load patients and in chronic cases of tuberculosis may provide valuable insight into their possible role in disease progression.

Keywords: TB: PPD; Ag 85 complex; PTB; HHC.

Introduction

Tuberculosis (TB) is an infectious disease caused by the bacillus *Mycobacterium tuberculosis*. It typically affects the lungs (pulmonary TB) but can affect other sites as well (extra pulmonary TB). The disease is spread in the air when people who are sick with pulmonary TB expel bacteria, for example by coughing. In 2015, 6.1 million new TB cases were notified to national authorities and reported to WHO. Notified TB cases increased from 2013–2015, mostly due to a 34% increase in notifications in India. WHO revised the Global TB Report 2016 and found that substantially upwards the estimates for India compared with earlier in 2011–2015 and observed the TB burden in India is higher than was thought earlier [1]. A serological test based on the detection of circulating antibodies against *M. tuberculosis*-

specific antigens could be a good alternative of therapy status during chemotherapy. Since early diagnosis and efficient treatment with therapy status are the basis for interrupting the transmission chain of TB and for controlling disease. A possibility for the development of more specific tests involves *M. tuberculosis* antigens that are absent in *Mycobacterium bovis* BCG vaccine strain and in environmental mycobacteria.

The Ag 85 complex consists of the major secretion products of MB BCG strain and has 3 major components: 85A (31 kDa), 85B (30 kDa), and 85C (31.5 kDa). This complex is strongly immunogenic and has been used for the development of assays to diagnose TB. However, low sensitivity was reported from studies using Ag85 in ELISA format and attributed to false-positive reactions caused by infections with environmental mycobacteria [2-4]. The transfer and subsequent transesterification is mediated by three well-known immunogenic proteins of the antigen 85 complex [5]. Besides, it can help in the study of immunological response during infection. However, low sensitivity and low specificity to the antigens limit the application of serological tests for TB [6-7]. In leprosy, selective increases in IgG1 and IgG3 antibodies to *Mycobacterium leprae* across the disease spectrum and both subclasses showed a highly significant correlation with bacterial load in the patients [8]. A predominant IgG1 responses to ESAT-6 in TB patients and suggest the useful TB disease biomarkers in monitoring treatment success [9]. The present study was conducted to find out the Antigen 85 detecting specific IgG subclasses to this antigen in monitoring treatment success. In this study serum levels of IgG subclasses (IgG1, IgG2, IgG3, IgG4) antibody specific to antigen 85 complex were evaluated in patients with pulmonary tuberculosis with their PPD^+ healthy house hold contact. The effect of the TB chemotherapy on levels of anti-TB antibody response was analyzed. Thus the objective of the present study was to determine IgG subclasses antibody response in tuberculosis, in order to improve our understanding of host immune response and the role of IgG subclasses in disease status as reference chemotherapy and with their healthy individual.

Methods

Study subjects

Serum samples of confirmed pulmonary TB patients from the State Tuberculosis Demonstration center (STDC), Agra were selected. All these patients were examined microbiologically (Ziehl Nielsen staining/culture) and who had undergone clinical and chest X-ray examinations prescribed by the categorized as per the guideline of Revised National TB Control program, Central TB Division, Government of India were included in the study. For the antibody (IgG Subclasses) study, patients with pulmonary TB were divided into four groups in accordance with the stage of treatment: (i) patients with

active TB (P 0 DAY), without prior treatment and Healthy house hold contact (HHC 0 DAY) (ii) patients with 2 months of anti-TB chemotherapy (P 2MF) and Healthy house hold contact (HHC 2MF) (iii) patients who had completed 6 months of treatment (P 6MF) and Healthy house hold contact (HHC 6MF) (iv) patients who had stop treatment before 3 months (P 9MF) and Healthy house hold contact (HHC 9MF). All the patients and Health house hold contact included in the present study were over 18 and under 50 years of age. Number of subject and their descriptive statistics for antigen 85 complex are given in Table 1. The study was approved by Institutional ethical committee and after gave informed consent for blood sampling after written information was provided**.**

Table 1. Study subjects for IgG subclasses in the study.

Study subject	**Total No.** **P 0 Day**	**Sex**		**Mean age Years**	**2 MF**	**6MF**	**9 MF**
		Male	**Female**				
Patients	67	35	32	27.76 ± 9.93	46	42	23
HHCs	45	20	25	34.40 ± 9.33	28	25	10

Antigens

Purified ESAT-6 and Antigen 85 complex were obtained from Colorado State University (Colorado USA) through a TB Research Material and vaccine Testing contract (NIH Contract HHSN 266200400091C/ADB Contract NOI-AI-40091.

Enzyme linked immunosorbent assay

Polystyrene 96-well micro plates were coated overnight with 100 µl per well of antigen 85 complex (25 ng/ml) diluted in carbonate bicarbonate buffer (pH 9.6) and then washed with PBS containing 0.05% Tween 20 (PBS-T). The remaining protein-binding sites were blocked in the coated wells by adding 200 µl blocking buffer, 2% BSA in PBS per well. Serum samples, previously collected (from consecutive patients and their healthy house hold contact within the recruitment period) and stored at -20^{o}C, were thawed and added in duplicates (100 µl per well) that were diluted 1: 100 in 1% BSA PBS-T. Hundred micro liters of 1% BSA PBS-T was used as negative control. After 2 hour incubation at 37^{0}C, plates were washed and mouse anti-human IgG1 (1:1000), IgG2 (1:2000), IgG3 1:4000), and IgG4 (1:1000) (Southern Biotechnology Associates, Inc., Birmingham, AL, USA) conjugated with HRP were added. Plates were incubated at 37^{0}C for 1 hour and washed, and a

substrate solution containing 0.5 mg /ml ortho-phenylene diamine in distilled water with 5 µl/ml H_2O_2 was used and kept at room temperature for 20 min in the dark. The reaction was stopped by adding 50 µl 7% H_2SO_4 each well, the optical density measured at 492 nm (Spectramax-M2 Reader, Molecular Devices, Sunny vale, CA, USA).

The results were expressed by ELISA index calculated by the formula-

EI = S/(B+3SD),

Where S is the average optical density value of the duplicate test sample and B was corresponds to the average optical density value of the duplicate PPD negative controls plus three times the SD.

Statistical analysis

All statistical tests were performed with Prism 3.02 (Graph Pad). Non parametric tests were used throughout when multiple comparisons were made for data set, One-Way analysis of variance (ANOVA) ($p < 0.05$) was used to test different among different participant group, the Kruskal-Wallis test with Dunn's Multiple Comparisons test was used. The data analysis was performed using nonparametric Mann-Whitney tests was used to test for different between pretreatment and treatment in the same patients and differences between the results obtained were considered significant if $P < 0.05$.

Results

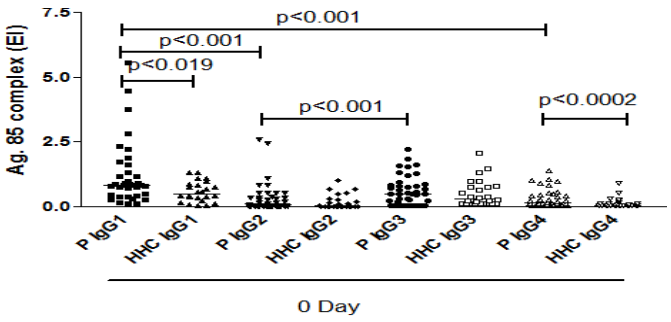
Detection of IgG subclasses against Antigen 85 complex in active tuberculosis and after chemotherapy.

In this study, serum levels of IgG1, and IgG4 antibody against antigen 85 complex were shown to be significantly elevated in sera of patients with active TB as compare to their HHC group respectively $p < 0.019$, $p < 0.0002$, . The result in Fig.1 A shown that the serum levels of specific 85 complex IgG1 is significantly higher in patients during initiation of treatment as compare to IgG2 & IgG4 ($p < 0.001$, $p < 0.001$ respectively) and serum levels of IgG3 was higher as compare to IgG2 ($p < 0.001$) (Fig 1 A). After 2 month therapy we observed significantly higher levels of IgG1 anti 85 complex antibody in patients as compare to their HHC and patients IgG2, IgG3, IgG4 respectively $p < 0.0036$, $p < 0.001$, $p < 0.001$, $p < 0.05$, (Fig.1 B). and after six month chemotherapy significant higher levels of IgG1 ($p < 0.006$) & IgG4 ($p < 0.004$) were observed as compare to their HHC (Fig 1 C) and these IgG1 were significantly higher as compare to their subclasses IgG2 and IgG4 against 85 complex respectively ($p < 0.01$, $p < 0.05$) (Fig.1 C) and after three month stop the treatment having observed elevated levels of IgG3 antibody against 85 complex as compare to their HHC ($p < 0.0058$) (Fig.1 D).

No significant differences were seen during the chemotherapy with IgG1 ($p > 0.05$). We further determine the comparative study of the IgG subclasses with therapy having observed elevated

levels of IgG2 in 9 month as compare to initiation of treatment ($p < 0.025$) and their, 2 month ($p < 0.0013$) and 6 month therapy ($p < 0.0153$) (Fig.2 B). We found levels of IgG3 anti 85 complex specific antibody were higher in patient during initiation of treatment as compare to after 2 month and 6 month therapy respectively $p < 0.032$, $p < 0.012$ and these depleted levels significantly higher after 3 month to stop the treatment $p < 0.011$ (Fig. 2 C). We observed significant higher level of IgG4 in 9 month follow up among to initiation of treatment & 2 month and 6 month therapy respectively $p < 0.0021$, $p < 0.0025$, $p < 0.002$ (Fig. 2 D).In our study also we have s een the average ELISA INDEX (EI) of IgG1, IgG2, IgG3, IgG4 during chemotherapy in which IgG1, IgG4, in patients were higher during 2 month as compare to initiation of treatment but decreased after 2 month therapy and again high after 3 month stop the treatment and the levels of IgG2 & IgG3 also decreased during therapy and increased again these levels to stop treatment after 3 months (Fig. 3).

A



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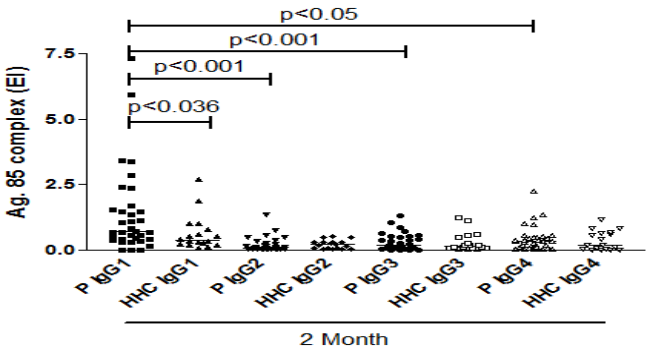

C

D

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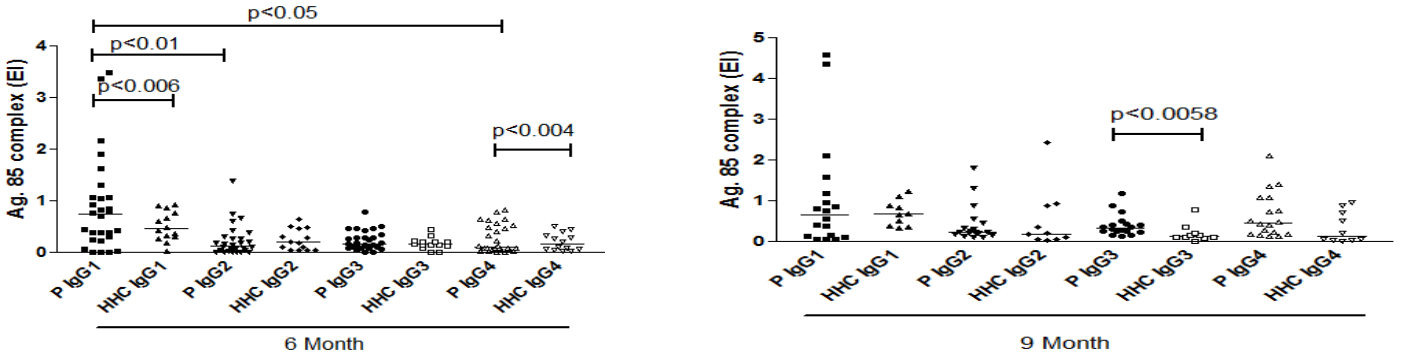
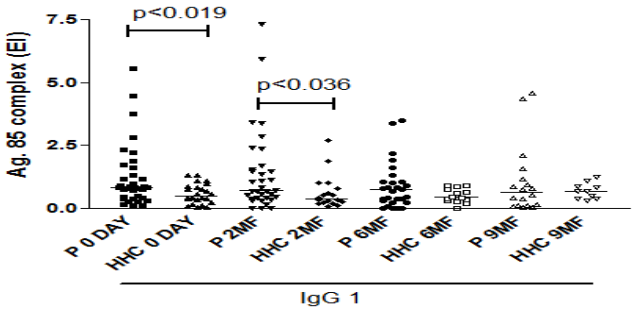


Fig 1. Levels of serum IgG1, IgG2, IgG3 and IgG4 against antigen 85 complex in patients with pulmonary TB and their Healthy House hold Contact (HHC). (A) Levels at 0 day = initiation of treatment and HHC (B) Levels after 2 Months of treatment and HHC. (C) Levels after 6 months of treatment and HHC. (D) Levels after 9 month follow up (after 3 month stop the treatment) and HHC. Bar represent the median.

A



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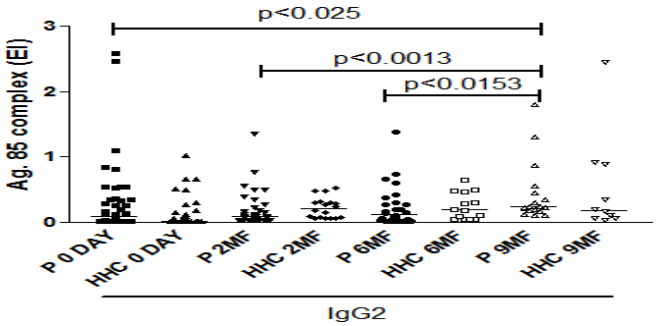

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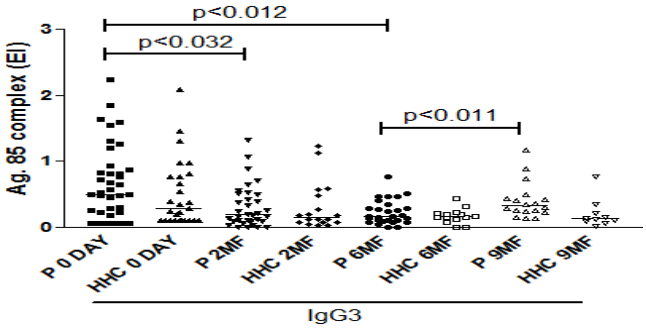

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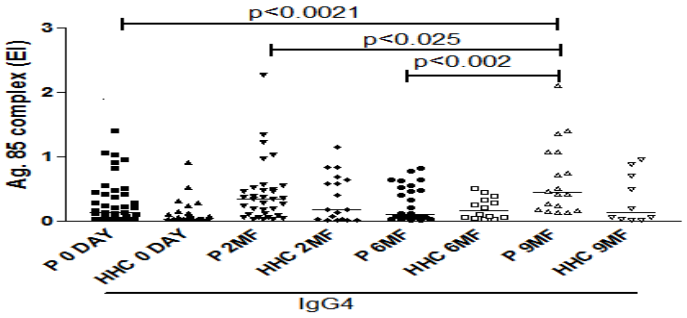

Fig- 2. Levels of serum IgG1 (A), IgG2 (B), IgG3(C) and IgG4 (D) against antigen 85 complex in patients with pulmonary TB and their Healthy House hold Contact (HHC) at 0 DAY, 2 Months and 6 Months of treatment and HHC and 9 month follow up. Bar represent the median.

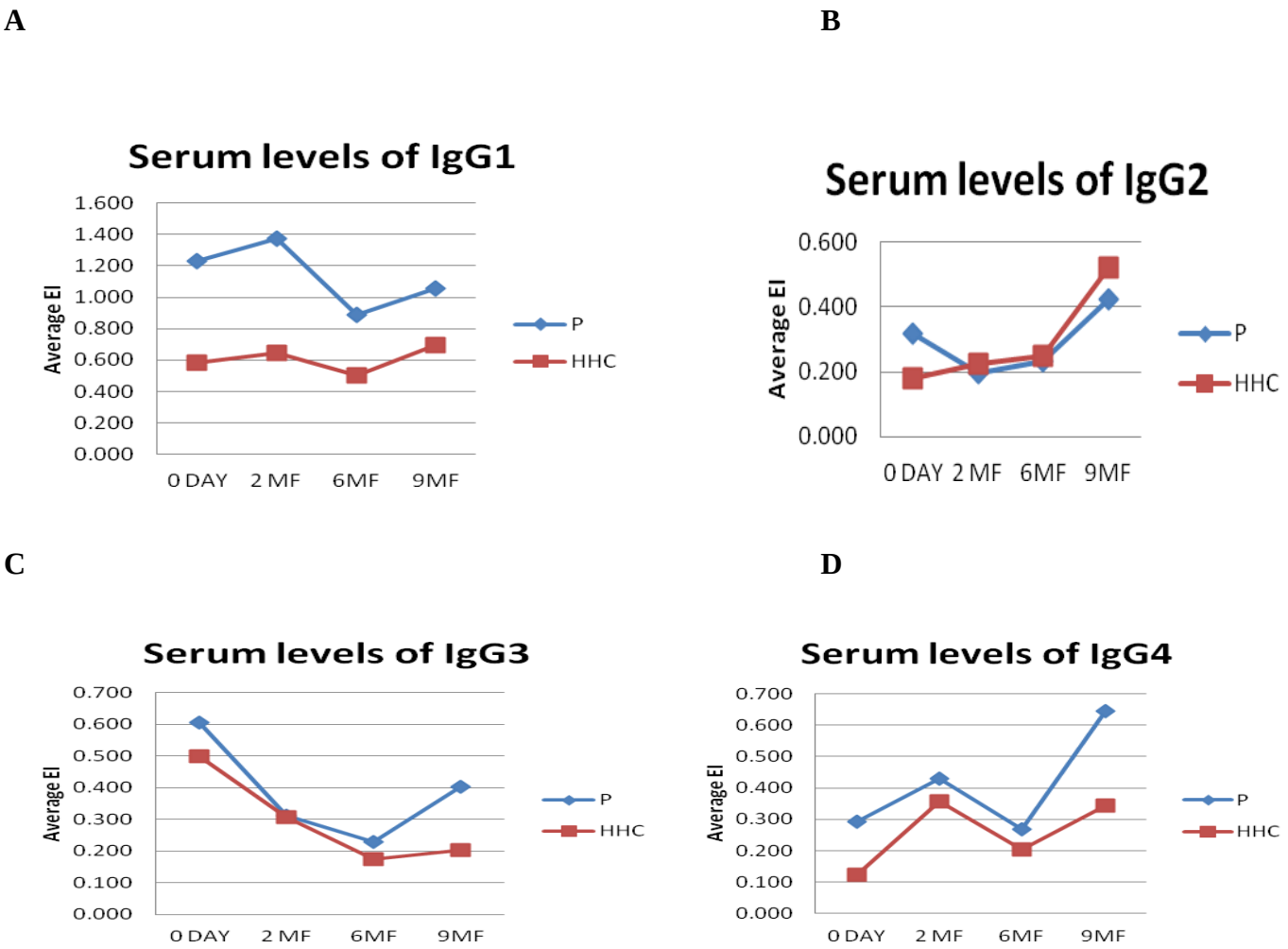

Fig -3. Line diagram showing Average **ELISA INDEX (EI) o**f serum (A) IgG1 (B) IgG2 (C) and IgG3 (D) IgG4, against Ag 85 Complex in patients with pulmonary TB and their Healthy House hold Contact (HHC).

Table 2. Cut off, sensitivity, specificity, LR+ and LR- for IgG subclasses against 85 complex by ROC analysis

	Cutoff	Sensitivity (%)	Specificity (%)	LR+	LR-	ROC area
IgG1	0.194	65.31	68.39	2.0597	0.5080	0.6814
IgG2	0.016	51.28	53.13	1.094	0.9170	0.5064
IgG3	0.037	55.56	60.47	1.4052	0.7350	0.5528
IgG4	0.0185	55.36	60.47	1.4002	0.7383	0.5866

(A) The cutoff was determined based on the ROC curves analysis and the point which showed the best accuracy (correctly classifying individual to their groups) was taken.

(B) The values of sensitivity and specificity were selected on the basis of cutoff as described above.

(C) +LR and -LR = positive and negative likelihood ratio (LR).

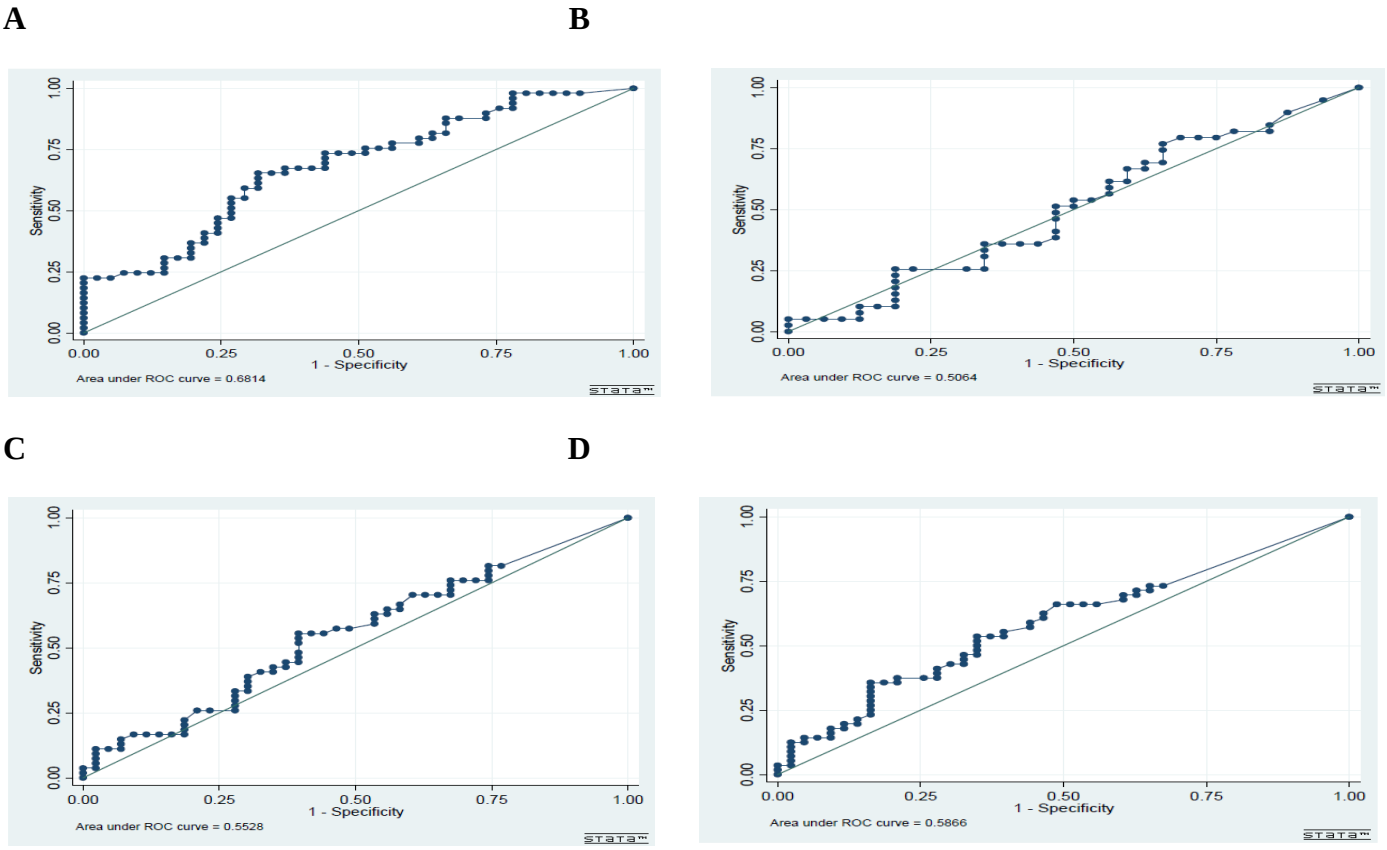

Figure 4. ROC curves for antigen 85 complex. (A) IgG1, (B) IgG2, (C) IgG3, (D) IgG4.

For the analysis of sensitivity and specificity of IgG subclasses against ESAT-6 and Ag 85 complex ROC analysis (Fig.4) was done. Area under the curve (AUC) for IgG1, IgG2, IgG3 and IgG4 responses against antigen 85 complex 0.6814, 0.5064, 0.5528, and 0.5866 respectively. Sensitivity and specificity was evaluated by ROC curve taking optimal cutoff point showing accuracy. Further, sensitivity for Ag85 complex specific IgG1, IgG2, IgG3 and IgG4 was 65.31%, 51.28%, 55.56% and 55.36%, respectively and specificity 68.39%, 53.13%, 60.47% and 60.47 % (Table 2) respectively.

Discussion

The most important results obtained from this study indicate the following: (i) patients with active TB present high serum IgG1, IgG4 against

antigen 85complex (ii) depleted levels of IgG2, IgG3 were seen after chemotherapy against antigen 85 complex. Several studies have detected antibodies in sera of patients with active TB against a variety of *M. tuberculosis* antigens [7, 10-12].

Reported elevation of antigen-specific IgG1 and IgG3, and the strongest association of IgG1 antibodies was observed with bacterial load rather than cellular responses [8]. Murine IgG2a and IgG2b and human IgG1 and IgG3 share the ability to fix complement and bind to protein antigens [13]. Murine IgG1 and human IgG4 are considered to be similar because of their property of binding to mast cells. Murine IgG3 and human IgG2 both recognize predominantly carbohydrate epitopes.

Observed the highest sensitivity (84.0%) and 85.2% specificity using Ag85 complex [13]. In an earlier report, Ag85 complex was reported to be 72% sensitive and 100% specific in Mexican Totonac Indians with pulmonary tuberculosis [14]. Similar findings have been confirmed by others [15]. Furthermore, we observed high antibody (60%) against Ag85 complex in patients undergoing anti-TB treatment, which suggests persistence of this antigen for longer duration. A report also indicates the immuno-dominant nature of Ag85 complex [16]. Found increased activation of B-cells is present following successful TB treatment, and that the expression of FASLG and IL5RA could potentially be utilised as a signature to monitor treatment [17]. Observed anti-PPD IgG2 could represent a fast and effective diagnostic algorithm for improving the diagnosis previous to obtain culture results [18].

Higher levels of antigen 85 complex IgG3 were observed during the initiation of treatment as compare to after chemotherapy and also observed significant higher levels of antibody after 3 month stop the treatment, these Elevated levels of antibodies against *M. tuberculosis* antigens with chemotherapy have been associated with intense stimulation of the humoral response by antigens released from killed bacteria combined with the disappearance of circulating Mycobacterial antigens so that specific antibodies are no longer trapped in immune complexes [19]. In this context, large amounts of IgG3 antibodies against the secreted antigen 85 complex antigens appear to be associated with viable and metabolically active bacilli. Study speculate that chemotherapy kills bacteria leading to release of a great amount of cytosolic antigens increasing specific antibody levels to antigen 85 complex antigens after the first term of treatment. One explanation might be large load of bacteria. The answer this question was obtained by our follow up study of patients with chemotherapy, and the observation that the cure of the disease restores the response to against 85 complex.

Little attention has been given to research into the subclasses of antibodies involved in TB [8-9, 20]. In this study, the analysis of IgG subclasses antigen 85 complex revealed a predominance of

IgG3 antibodies against ESAT-6 in relation to IgG1, IgG3 and IgG4 and IgG1 against antigen 85 complex with reference to other subclasses in serum of patients with active TB. Our results are consistent with other studies that also observed predominance of IgG1 antibodies in the serum of patients with TB [8, 20]. Although Ag85 complex is reported to be cross-reactive with other mycobacteria as well [21].

The detection of serum IgG1 and IgG3 antibodies specific to antigen 85 complex may represent an additional tool in the diagnosis of active TB when compared with PPD^+ healthy house hold contact and, together with IgG2 and IgG4 detection specific to both the antigen, may be useful as a biomarker of treatment success when related to their pre-treatment values and should be investigated further with their T cells response.

Conclusion

Developing countries needs a specific diagnostic test that should be easy to perform with limited cost. The selection of antigen like Ag85 complex to be strong targets for humoral and cell-mediated response with TB vaccine strategy. The detection of serum IgG subclasses specific to Ag85 complex antigens may represent an additional tool in the diagnosis of active and their therapy status.

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