Original Article

Elisa Method for Evaluation of Anti A60 IgG for the Diagnosis of Tuberculosis

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ABSTRACT

BACKGROUND/OBJECTIVES: An ELISA test based on mycobacterial antigen A60 for the estimation of Anti (A60) IgG in the serum has been used successfully for the rapid diagnosis of tuberculosis in studies done in the European countries, China and India. To demonstrate and evaluate anti-tuberculosis anti-body IgG for rapid diagnosis of both pulmonary and extra pulmonary tuberculosis, this study was carried out in our set up.

METHODS: ELISA assay based on mycobacterial antigen A60 (Anda Biologicals, France) was used to the sera obtained from 69 cases of tuberculosis and 136 controls in the population of Karachi, Sindh -Pakistan.

RESULTS: Of 136 controls, only 17.7% were positive for IgG antibodies and showing 82.3% specificity. A very high sensitivity (88.8%) was seen in sputum positive active pulmonary tuberculosis. Relatively low (75%) sero-positivity was seen in cases of sputum negative active pulmonary tuberculosis compared to those of sputum positive active pulmonary tuberculosis. In cases of extra pulmonary tuberculosis, 47.3% sensitivity was observed. In case of healed tuberculosis quite a large number of cases was found positive for IgG (62.5%).

CONCLUSION: The estimation value of anti tuberculosis antibody IgG against A60 for the rapid diagnosis is proved from our data. Considering all the cases of active tuberculosis and the controls the global sensitivity of 69.8% and specificity of 82.4% were obtained when IgG antibodies estimation were taken into account.

Key Words: Tuberculosis. Sensitivity. Specificity. Diagnosis. Antibody IgG.

INTRODUCTION

Pakistan is ranked 8th in terms of estimated number of Tuberculosis cases by WHO in 22 high burden countries.[1] Almost 1.5 million people suffer from Tuberculosis in this country of 144 million population indicating a prevalence exceeding 1% of the total population.[2] Global tuberculosis report by WHO mentions the case notification rate for Pakistan as 23/100,000 in the year 2001.[3] Tuberculosis has become an important public health problem in today's world whereas previously it was considered a nuisance associated usually with the developing countries.[4] A recent increase in tuberculosis incidence and complications has been registered in connection with the spread of antibiotic resistance and AIDS.[5] The diagnosis of mycobacterial disease depends upon identifying the infective organism in secretion or tissues of diseased individual. There are several limitations of this method of diagnosis.[6] The rapid detection and identification of Mycobacterium Tuberculosis complex in samples are extremely important for optional diagnosis, and effective treatment as well as for prevention and control of tuberculosis transmission.[7] Sero-diagnostic tests based on the presence of antibodies against mycobacterial antigens in the sera have been identified, purified and tested, with various degrees of success.[8] The present study was designed to demonstrate and evaluate A-60 specific IgG antibody levels in the sera for the rapid diagnosis of different clinical forms of tuberculosis.

MATERIAL AND METHODS

This study was conducted in the Department of Microbiology of Basic Medical Sciences Institute (BMSI), Jinnah Postgraduate Medical Centre (JPMC) Karachi, Sindh - Pakistan. Patients with tuberculosis and non-tuberculosis patients were selected from different medical and surgical wards of JPMC. Serum IgG against antigen A 60 was estimated in 69 patients of tuberculosis and 136 controls with their age ranging from 13-65 years. The diagnosis of tuberculosis was based on clinical and radiological criteria, histopathology, presence of Acid Fast Bacilli (AFB) and clinical

response to anti-tuberculosis treatment. The laboratory tests included estimation of ESR, Haemoglobin%, TLC and DLC by standard techniques. Radiological investigations included sky gram of chest PA view in all patients. Sputum for presence of AFB was recorded in all patients of pulmonary pathology. Mantoux test was recorded in healthy normal subjects. Serum samples from patients with tuberculosis, non-tuberculous patients and normal healthy subjects were collected and frozen at -20°C after proper labeling. Concentration of A60 specific IgG in the sera for cases of human tuberculosis and controls were measured by indirect ELISA technique. The distribution of patients with tuberculosis and controls was as follows:

Tuberculosis Patients:

This group (69 patients) was classified[5] into the following categories;

1. Patients with healed tuberculosis (16 cases). These subjects comprised of cases of pulmonary TB who had been given anti-tuberculous therapy (ATT) for 9 months. They were all clinically healed at the time of study. Two of them completed their treatment recently. The rest of the patients had taken ATT 2 to 10 years back.

2. Patients with sputum positive active pulmonary tuberculosis (18 cases). They had tubercle bacilli in their sputum and with clear roentgenogram evidence.

3. Patients with sputum negative active pulmonary tuberculosis (16 cases). The diagnosis was based on clinical and radiological data but AFB were absent in sputum specimens.

4. Patients with extra pulmonary tuberculosis (19 cases). This group comprised of pleural (4 cases), lymphonodal (5 cases), tuberculosis meningitis (2 cases), abdominal (2 cases), osseous (4 cases), miliary (1 case) and psoas abscess (1 case).

Control Group:

The control group (136 subjects) was classified into the following categories:

1. Tuberculosis negative healthy subjects (15 subjects). They were included into this group according to negative response to intra-dermal injection of 51 U of PPD. A diameter of induration less than 10 mm after 72 hours was considered as negative test.

2. Tuberculosis positive healthy subjects (9 subjects). They were included into this group accord-

ing to positive response to intra-dermal injections of 5I U of PPD. A diameter of induration of >10mm after 72 hours was considered as positive test.

3. Non-tuberculosis patients with pulmonary pathology (15 subjects). They included 3 cases of lung abscess, 2 cases of COPD, 3 cases of broncho-genic carcinoma and one case of each chronic bronchitis, hydro-pneumothorax, nephritic syndrome with pleural effusion and easinophylic pneumonia.

4. Non-tuberculosis patients with extra pulmonary pathology (7 subjects). They included 5 cases of cervical lymph-adenopathy and one case of each nephritic syndrome and thyroid benign pathology.

5. Contact cases of tuberculosis (36 subjects). Thy included members of the staff serving in the wards like doctors, nurses, ward-boys, ayahs, dieticians, waiters, dressers, liftmen, sweepers etc.

6. Subjects handling mycobacteria (54 subjects). They included technicians from different laboratories in the town.

Laboratory Procedures:

Specimens of blood were taken with appropriate consent. Five ml of blood was drawn from superficial vein from each subject with the help of disposable syringe under aseptic conditions. It was transferred to a sterile cup and allowed to clot at room temperature. Then it was centrifuged and serum was separated and transferred with the help of disposable Pasteur pipette to a sterile cup and stored in a refrigerator at -20° C until processed for analysis. In the present study the measurement of IgG antibodies against A 60, strain BCG of M. bovis was done in serum of our study population with an ELISA KIT (Anda TB Biological, Strasbourg, France). Stored serum samples of our study population were taken out from the freezer one-hour prior to the test. Anti A60 IgG were estimated in the sera of the subjects under study employing indirect ELISA technique as per recommendations of the manufacturer. Each time the positive as well as the negative reference sera provided with diagnostic kit were included in the test along with the test sera. For IgG determination, the curves were constructed by plotting the optical density values of different reference curve.

Principle of Method:

Anda TB is an immune-enzymatic test with dosage on a solid phase. Sample of human sera is distributed in the wells of micro-titration plate coated with the A60

mycobacterium complexes. Their incubation allows the formations of antigen-antibody complexes. Washing eliminates the unloading components of the sera. The wells are thereafter incubated with peroxides labeled antihuman IgG antibodies that bind to the antibody complexes present. The unbound components are eliminated by washing. The peroxidase substrate, tetra-methyl benzidine (TMB) containing hydrogen peroxide, is thereafter introduced in the wells. A color develops during the reaction of peroxide with TMB, whose intensity is proportional to the quantity of specific antibodies present in the sample.

RESULTS

The present study involved analysis of 205 serum samples for estimation of IgG against antigen A 60 for rapid diagnosis of tuberculosis by ELISA technique from subjects belonging to groups of tuberculosis patients and various control groups.
Serological Analysis of Tuberculous Group:
In serological analysis of tuberculous cases with regard to antigen A60 specific antibodies of IgG class, it was observed that very high sensitivity 88.8% (16/18) was seen in 18 cases of sputum positive active pulmonary tuberculosis. However, in 16 cases of sputum negative active pulmonary tuberculosis serological positivity was observed as 75% for IgG antibodies while 47.3% for IgG was observed in 19 cases of extra pulmonary tuberculosis. On the contrary, 16 cases of inactive tuberculosis depicted high positivity of IgG antibodies, considering the overall picture of active tuberculosis (69.8%) for IgG antibodies. (**Table I**) The corresponding mean titers for the class of IgG antibodies in active tuberculous cases were 1.255 optical density. (**Table II**)
Serological Analysis of Control Groups:
In serological analysis of controls with regard to antigen A60 specific antibodies of IgG class, it was observed that 15 tuberculin negative healthy subjects were negative for anti A60 IgG indicating 100% seronegativity in this group. This specificity was observed at 1:100 serum dilutions. They were healthy at the time of collection. Among the tuberculin positive healthy controls, 33.3% (3/9) were positive for IgG antibodies. Overall among healthy controls very few subjects yielded a positive serology; 12.5% (3/24) being positive for IgG. There was slight serological difference between tuberculin positive and tuberculin

negative control. However, when the 54 laboratory technicians were analyzed, 14.8% (8/54) were positive for IgG antibodies. Among 36 contact cases, very few subjects yielded a positive serology; only 13.8% (5/36) were positive for IgG. Among the 22 diseased controls, 36.3% (8/22) were positive for IgG antibodies. Taking the overall picture of 136 controls, 17.7% (24/136) were positive for IgG antibodies (**Table III**). The corresponding mean titres for IgG antibodies in control subjects were 0.601 OD for IgG. (**Table IV**)

TABLE I:
SEROLOGICAL ANALYSIS OF CASES OF TUBERCULOSIS

Subjects	Number of cases	A60 ELISA Sensitivity IgG Number (%)
Patients with sputum positive active pulmonary tuberculosis	18	16(88.8)
Patients with sputum negative active pulmonary tuberculosis	16	12(75.0)
Patients with extra pulmonary tuberculosis	19	09(47.3)
Patients with healed tuberculosis	16	10(62.5)
Total	69	37(69.8)

TABLE II:
A60 SPECIFIC ANTIBODY TITRES IN CASES

Subjects	Number of cases	Mean antibody titers (Range) IgG
Patients with sputum positive active pulmonary tuberculosis	18	1.683 (0.623-2.587)
Patients with sputum negative active pulmonary tuberculosis	16	1.307 (0.358-2.802)
Patients with extra pulmonary tuberculosis	19	0.776 (0.281-2.024)
Patients with healed tuberculosis	16	1.126 (0.358-2.802)

TABLE III:
SEROLOGICAL ANALYSIS OF NON-TUBERCULOUS INDIVIDUALS (CONTROLS)

Control Groups	Number of cases	A 60 ELISA Specificity IgG Number (%)
Tuberculin negative healthy subjects	15	0(100%)
Tuberculin positive healthy subjects	09	03(66.6%)
Non-tuberculous patients with Pulmonary pathology	15	05(66.6%)
Non-tuberculous patients with extra pulmonary pathology	07	03(68.2%)
Contact of cases of tuberculosis	36	05(87.2%)
Subjects handling mycobacteria	54	08(85.2%)
Total	136	24(82.4%)

TABLE IV:
A60 SPECIFIC ANTIBODY TITRES IN CONTROLS

Subjects	Number of cases	Mean antibody titers (Range) IgG
Tuberculin negative healthy subjects	15	0.416 (0.293-0.549)
Tuberculin positive healthy subjects	09	0.700 (0.233-0.549)
Non-tuberculous patients with pulmonary pathology	15	0.684 (0.265-2.072)
Non-tuberculous patients with extra pulmonary pathology	07	0.798 (0.242-1.683)
Contact of cases of tuberculosis	36	0.527 (0.262-0.990)
Subjects handling mycobacteria	54	0.522 (0.210-1.738)

DISCUSSION

The present work was performed to demonstrate and evaluate the IgG antibodies against A60 antigen by ELISA technique for the rapid serological diagnosis of tuberculosis. A cut off point which is essential for the interpretation of serological data, is based on large surveys of controls subjects (healthy persons with no history of a given disease and patients with other diseases) and varies according to environmental conditions. Our approach was to apply the cut off recommended by the manufacturer i.e. IgG absorbance (sero units), negative (<125), dubious (125 – 225) and positive (>225). In this study, we estimated IgG against antigen A 60 in control groups and patients from different forms of tuberculosis. The usefulness of studying several control groups consists both in defining the cut off point to use for tuberculous patients from our region and consequently, evaluating the specificity of the test. In the healthy control groups, very few subjects yielded a positive serology against A60 antigen. In tuberculin negative healthy subjects, all were serological negative for IgG class with 100% sero-negative. Whereas, among tuberculin positive healthy subjects, 3 were positive for IgG. In human contacts with cases of tuberculosis, a few subjects showed a positive A60 serology, and only 13.8% were positive for IgG antibodies. In the group of laboratory staff who had been routinely handling the mycobacterial cultures showed surprisingly the low serological positivity. Only 14.8% were positive for IgG antibodies. Earlier reports and sero-positivity against A60 antigen in the control population ranged between 0-50% for IgG antibodies. The IgG levels in the control subjects in this study were appreciably lowered compared to those in case of active and healed tuberculosis. Similar results were observed in India.[9] These findings are more or less in the agreement with earlier reports.[10-12] In this study, specificity of the tests ranged from 66.6% to 80% in non-tuberculosis pulmonary pathology, but some patients analyzed departed strikingly from this norm and yielded different patterns of sero-positivity. Other authors have also reported similar events.[10,11,13-15] A very good serological response was observed in cases with sputum positive active pulmonary tuberculosis with regard to IgG antibodies depicting a sensitivity of 88.8% and the mean levels of IgG antibodies were appreciably higher than in the controls. In studies carried out earlier, an IgG sensitivity ranging from 48-100% has been documented.[9,11,12] These wide variations could be due to different age groups studied, geographical area and severity of disease in different studies as well as variety in cut off limit used. In the present study, relatively low sero-positivity was seen in cases of sputum negative active pulmonary tuberculosis compared to those of sputum

positive active pulmonary tuberculosis as shown in a previous study.[11] As far as serology in patients with extra pulmonary tuberculosis is concerned, a sensitivity of 47.3% for IgG was observed. The mean antibody levels were lower than the active pulmonary tuberculosis. A few studies done in Western countries have shown 100% sensitivity of IgG in cases of extra pulmonary tuberculosis.[8, 9] In an Indian study, IgG with 73.8% sensitivity was observed in cases of extra pulmonary tuberculosis. Again wide variations were observed which may be due to different clinical type of extra pulmonary tuberculosis, variety in cut off limit used, different age groups studied, and demographic areas as well as severity of diseases in different studies. On analysis of cases of healed tuberculosis (treated) quite a large number was found positive for IgG (62.5%); the results observed in cases of healed tuberculosis are comparable to those documented by previous study.[9] Moreover, the mean antibody levels were also higher than those of control, although the levels were lower than those in active pulmonary tuberculosis. Substantial lowering of anti A60 immunoglobulins observed by different authors is probably due to the varying time between the inclusion in the study and the end of anti-tuberculosis therapy.[15] In this study, detection of anti A60 antibodies in patients with pulmonary and extra pulmonary tuberculosis was negative in a very small percentage of cases. This may be a result of immuno-depression due to disease as well as to the presence of immune complex.[15]

In this study, a small percentage of healthy subjects and patients with non-tuberculous disease showed sero-positivity. This might be due to either sub-clinical infection of environmental non-tuberculous mycobacteria that also express A60 or to the presence in the host of commensal non-pathogenic mycobacteria. The deregulation of the humoral immune response that occurs frequently in several diseases might be another cause of positive results in patients with non-tuberculous disease.[16] The estimating of IgG antibodies against A60 antigen for rapid diagnosis of pulmonary and extra pulmonary tuberculosis is clear from our data. Considering all the cases of active tuberculosis and the controls the global sensitivity of 69.8% and specificity of 82.4% were obtained when IgG antibodies estimations were taken into account. In India, test sensitivity of 91.6% and specificity of 90% were demonstrated in post primary tuberculosis by estimating A60 specific IgG antibodies.[9] Similar results were observed in France where a test sensitivity of 98.6% and specificity of 86.7% was reported.[11] Sero-diagnostic

tests based on the presence of antibodies against mycobacterial antigens in sera have been described with various degrees of success.[8]

CONCLUSION

The present study has confirmed the value of estimation of anti-tuberculosis antibody IgG against A60 for the rapid diagnosis of tuberculosis. Considering all the cases of active tuberculosis, the global sensitivity of 69.8% and specificity of 8.4% were obtained but the sensitivity in extra-pulmonary tuberculosis was only 43.7%. This test should not be considered to be diagnostic tool by itself. It should be used in conjunction with other diagnostic mean that together allow the determination of a diagnosis.

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