

EVALUATION OF ANTI A60 IgM FOR THE DIAGNOSIS OF TUBERCULOSIS WITH ELISA METHOD

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ABSTRACT:

Evaluation of Anti Tuberculous IgM antibodies for rapid diagnosis of both pulmonary and extra-pulmonary tuberculosis. ELISA assay based on mycobacterial antigen A-60 (Anda Biologicals, France) was used on the sera obtained from 69 cases of Tuberculosis and 136 controls in the population of Karachi, Pakistan. Of 136 controls only 21.3 % were positive for IgM antibodies and showing 78.7% specificity. A very high sensitivity 72.2% was seen in sputum positive active pulmonary tuberculosis. Relatively low 56.2% 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 57.8 % sensitivity was observed. In cases of healed tuberculosis only 18.7% were found positive for IgM. The estimation value of IgM against A-60 for the diagnosis of tuberculosis is proved from our data. Considering all the cases of active tuberculosis and the controls the global sensitivity of 62.2% and specificity of 78.7% was found when IgM estimation was taken into account.

Keywords: Tuberculosis, Antigen A-60, Antibody IgM, Serological analysis, Diagnosis.

INTRODUCTION

Tuberculosis is one of the most common infectious diseases that infect about two million people of the world (Ghadiri *et al.*, 2008). Pakistan is ranked 6th in terms of estimated number of tuberculosis cases by WHO in high burden countries (Khan *et al.*, 2006). Global tuberculosis reports by WHO mentions the case notification rate for Pakistan as 23/100,000 in the year 2001(WHO, 2003). 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 (Qazi & Khan, 1998). A recent increase in tuberculosis incidence and complications has been registered in connection with the spread of antibiotic resistance and AIDS (Coetsier *et al.*, 1994).

The diagnosis of mycobacterial disease depends upon identification of the infective organism in secretions of diseased individual. There are several limitations of this method for diagnosis (Daniel & Debanne, 1987). The rapid detection and identification of Mycobacterium tuberculosis is a complex process. Samples are extremely important for optional diagnosis and effective treatment as well as for prevention and control of tuberculosis transmission (Shurji *et al.*, 2005). Sero diagnostic tests based on the presence of antibodies against mycobacterial antigens in the sera have been identified, purified and tested, with various degree of success (Hendrickson *et al.*, 2000). The present study was designed to demonstrate and evaluate A-60 specific IgM antibody levels in the sera for the rapid diagnosis of different clinical forms of tuberculosis.

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METHODS AND MATERIAL

This study was conducted in the Department of Microbiology of Basic Medical Sciences institute (BMSI), Jinnah postgraduate Medical Centre (JPMC) Karachi, Sindh, Pakistan. Patients were selected from different medical and surgical wards of JPMC. Serum IgM against antigen A60 was estimated in 69 patients of tuberculosis, 136 controls with their age ranges between 13-65 years. The diagnosis 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, Hemoglobin% TLC and DLC by standard techniques. Radiological investigation included X-ray chest PA view in all patients. Sputum for presence of AFB was recorded in all patients with 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 A-60 specific IgM 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:

69 patients were classified 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 two to ten 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), tuberculous 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 5 IU 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 according to positive response to intra-dermal injection of 5 IU of PPD. A diameter of induration of >10 mm 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 bronchogenic 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). They 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. 5 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 sterile cup and stored in a refrigerator at -20°C until process for analysis. In the present study, the measurement of IgM 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 A-60 IgM 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 negative reference sera provided with diagnostic kit were included in the test along with the test sera. For IgM 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 A-60 mycobacterium complexes. Their incubation allows the formation of antigen-antibody complexes. Washing eliminated the unbound components of the sera. The wells are thereafter incubated with peroxidase labeled antihuman IgM antibodies that binds 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 peroxidase 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 IgM 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 tuberculosis group:

In serological analysis of tuberculosis cases with regard to antigen A-60 specific antibodies of IgM class, it was observed that 72.2% (13/18) were positive, in 18 cases of sputum positive active pulmonary tuberculosis. However, in 16 cases of sputum negative active pulmonary tuberculosis serological positivity was seen as 56.2 % for IgM antibodies. 57.8% were positive for IgM in 19 cases of extra pulmonary tuberculosis. On the contrary 16 cases of inactive tuberculosis depicted a low positivity 18.7 % for IgM antibodies. Considering the over all picture of the case of active tuberculosis 62.2% were positive for IgM antibodies. (Table 1). The corresponding mean titres for the class of IgM antibodies in active tuberculosis cases were 1.216 OD (Table 2).

Serological analysis of control groups:

In serological analysis of controls with regard to antigen A 60 specific antibodies of IgM class, it was observed that 15 tuberculin negative healthy subjects were negative for anti A60 IgM, indicating 100% sero-negativity 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 IgM antibodies. Overall among healthy controls very few subjects yielded a positive serology: 12.5% (3/24) being positive for IgM antibodies. There was slight serological difference between tuberculin positive and tuberculin negative controls. However, when the 54 laboratory technicians were analyzed 18.5% (10/54) were positive for IgM antibodies. Among 36 contact cases, very few subjects yielded a positive serology: only 26.3% (10/36) were positive for

Table 1
Serological analysis of cases of tuberculosis

Subjects	Number of Cases	A-60 ELISA Sensitivity IgM Number (%)
Patients with sputum positive active pulmonary tuberculosis	18	13 (72.2)
Patients with sputum negative active pulmonary tuberculosis	16	09 (56.2)
Patients with extra pulmonary tuberculosis	19	11 (57.8)
Patients with healed Tuberculosis	16	03 (18.7)
Total	69	33 (62.2)

Table 2
A60 specific antibody titres in cases of tuberculosis

Subjects	Number of Cases	Mean antibody titres (Range) IgM
Patients with sputum positive active pulmonary tuberculosis	18	1.458 (0.816-2.484)
Patients with sputum negative active pulmonary tuberculosis	16	1.318 (0.284-2.390)
Patients with extra pulmonary tuberculosis	19	1.109 (0.388-1.884)
Patients with healed tuberculosis	16	0.732 0.263-1.554)

IgM antibodies. Among the 22 diseased controls 27.2% (6/22) were positive for IgM antibodies. Taking the overall picture of 136 controls 21.3% (29/136) was positive for IgM antibodies (Table 3). The corresponding mean titre for IgM antibodies in control subjects were 0.728 x OD (Table 4).

DISCUSSION

The present work was performed to demonstrate and evaluate the IgM antibodies against A-60 antigen by ELISA technique for rapid serological diagnosis of tuberculosis. A cut off point which is essential for the interpretation of serological data is based on large surveys of control subjects and varies according to environmental conditions. Our approach was to apply the cut off recommended by the manufacturer i.e, IgM absorbance, negative (< 0.8), dubious (0.8-1.0), Positive (> 1.0)

In this study for IgM antibodies, a cut off value of 1.00 of optical density index (ODI) appeared to give good result with global sensitivity of 62.2% and global specificity 78.6% (Table 3). In the present work we studied several control groups with healthy and pathological and evaluated separately patients suffering from different forms of tuberculosis. The usefulness of studying several control groups consists both in defining the cut off point to use for tuberculosis patients for our region and consequently, evaluating the specificity of the test.

In the healthy control groups very few subjects yielded a positive serology against A-60 antigen. In tuberculin negative healthy subject all were serologically negative for IgM antibodies with 100 % specificity. Whereas tuberculin positive healthy subjects, 3 subjects

Table 3
Serological analysis of non- tuberculosis individuals (controls)

Control Groups	Number of Cases	A-60 ELISA Sensitivity IgM Number (%)
Tuberculin negative healthy Subject	15	0 (100)
Tuberculin positive healthy subject	09	03 (33.3)
Non- tuberculosis patients with pulmonary pathology	15	03 (20.0)
Non-tuberculosis patients with extra pulmonary pathology	07	03 (42.8)
Contact of cases of tuberculosis	36	10 (27.7)
Subject handling mycobacteria	54	10 (18.5)
Total	136	29 (21.3)

Table 4
A-60 Specific antibody titres in controls

Subjects	Number of Cases	Mean antibody titers (Range) IgM
Tuberculin negative healthy subject	15	0.573 (0.302-0.873)
Tuberculin positive healthy subject	09	0.743 (0.279-1.389)
Non- tuberculosis patients with pulmonary pathology	15	0.886 (0.479-1.412)
Non- tuberculosis patients with extra pulmonary pathology	07	0.824 (0.386-1.453)
Contact of cases of tuberculosis	36	0.641 (0.238-1.573)
Subject handling mycobacteria	54	0.702 (0.313-1.989)

were positive for IgM. In human contacts cases of tuberculosis, few subjects showed a positive A-60 serology. Only 27.7% were positive for IgM antibodies. In the group of laboratory staff who had been routinely handling the mycobacterial culture showed surprisingly the low serological positivity. Only 18.5 % were positive for IgM antibodies. IgM levels in the control subject in this study were appreciably lower compared to those in cases of active and healed tuberculosis. Similar results were shown in India (Gupta *et al.*, 1995). The findings are more or less in the agreement with earlier reports (Baelden *et al.*, 1990; Charpin *et al.*, 1990; Gevaudan *et al.*,

1992; Kaustova, 1996). In this study seronegativity of the test was 80 % in non-tuberculous patients with pulmonary pathology and 57.1% in non-tuberculous patients with extra pulmonary pathology, but some patient analyzed departed strikingly from this norm and yielded different pattern of seropositivity. Other authors reported similar events (Baelden *et al.*, 1990; Charpin *et al.*, 1990; Kaustova, 1996; Gullea *et al.*, 1998, Alifano *et al.*, 1994).

A good serological response was observed in cases with sputum positive active pulmonary tuberculosis with regards to IgM

antibodies depicting a sensitivity of 72.2%, the mean level of IgM antibodies were appreciably higher than in the controls. In studies carried out earlier an IgM positive ranging from 5-76% has been documented (Gupta *et al.*, 1995; Charpin *et al.*, 1990; Gevaudan *et al.*, 1992). These wide variations could be due to different age groups studies, geographical areas and severity of disease in different studies as well as variety in cut off limit used.

In the present study, relatively low seropositivity was seen in cases of sputum negative active pulmonary tuberculosis compared to those of sputum positive active pulmonary tuberculosis as shown in previous study (Charpin *et al.*, 1990). As far as serology in patient with extra-pulmonary tuberculosis is concerned a sensitivity of 57.8% for IgM was observed. The mean antibody was lower than the active pulmonary tuberculosis.

In Indian study IgM 22.3% positivity was observed in cases of extra pulmonary tuberculosis (Hendrickson *et al.*, 2000; Gupta *et al.*, 1995). Again wide variations were observed which may be due to different clinical types 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 healed tuberculosis (treated) a small number were found positive for IgM 18.7%. The results observed in cases healed tuberculosis compared to those documented by previous study. Moreover the mean antibody were also higher than those of controls, although in levels were lower than those in active pulmonary tuberculosis. Substantial lower of anti A60 immunoglobulin were observed by different authors are probably due to varying time between the inclusion in the study and the end of anti-tuberculosis therapy.

In our 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

result of immunodepression due to disease as well as to the presence of immune complex (Alifano *et al.*, 1994).

In our study a small percentage of healthy subjects and patients with non-tuberculosis 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 disregulation of the humoral immune response that occurs frequently in several diseases might be another cause of positive results in patients with nontuberculous disease.

CONCLUSION

The present study has confirmed the value of estimation of anti-tuberculosis antibody IgM against A60 for the rapid diagnosis of tuberculosis. The estimating of IgM antibodies against A60 antigen for rapid diagnosis of pulmonary and extra-pulmonary tuberculosis is proved from our data. Considering all the cases of active tuberculosis and the controls the global sensitivity of 62.6% and specificity 78.7% were obtained when IgM antibodies estimation were taken into account. The time lapse between occurrence of symptoms and initiation of specific treatment must also be taken into account in evaluating serological data and diagnostic purposes because IgM antibodies are rapidly either replaced or else accompanied by an IgA or IgG surge when tuberculosis remain untreated.

This test should not be considered to be diagnostic tool by itself. It should be uses in conjunction with other diagnostic mean that together allow the determination of a diagnosis.

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