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Status of enzyme-linked immunosorbent assay test for tuberculosis serology in low socio economic status and undernourished children with suspected pulmonary tuberculosis

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ABSTRACT

Background: Tuberculosis (TB) continues to be a major health problem in developing countries like India. The diagnosis of childhood tuberculosis is very difficult, because of paucibacillary nature of childhood disease and the confirmation is the detection of *Mycobacterium Tuberculosis* from sputum and similar specimen i.e. lymph node or other body fluid. The other means of diagnosis determination of antibody of tubercular IgM/IgA/IgG by enzymelinked immunosorbent assay (ELISA). Once it was considered a very good one but subsequent analysis is not so specific. The sensitivity and specificity was not good. Thus the ELISA has been discarded by WHO in 2011. The sensitivity and specificity was done all the patients' irrespective age, nutritional and socioeconomic status. The objective of the study was to know its exact status of tubercular antibodies in low socioeconomic status.

Methods: The present study was conducted on 115 children who were suffering from clinically suspected pulmonary tuberculosis (PT) and those who had enlarged cervical lymph nodes as extra pulmonary tuberculosis (EPT). For all the cases MT test was done. The ELISA serological test of IgM and IgG antibodies were done.

Results: The present study has documented that the sensitivity and specificity is much lower than the studies conducted by the other authors conducted in different types of population of different age groups.

Conclusions: The ELISA serological tests of antibodies have false positivity and negativity. This leads over diagnosis or under diagnosis of tuberculosis. It is strongly recommended that these commercial tests not be used for the diagnosis of pulmonary and extra-pulmonary TB.

Keywords: ELISA test, Low socio-economic status, Tuberculosis, Undernourished children

INTRODUCTION

Tuberculosis (TB) is prevalent all over the world especially in under-developed countries. World Health Organization (WHO) had declared TB as a 'global TB emergency' in the year 1993. Paediatric TB remains a public health emergency. This is particularly evident in developing countries with poor public health

infrastructure. Despite longstanding efforts to conquer TB, it continues to be a major health problem in developing countries like India. The mainstay for its control is the rapid and accurate identification of infected individuals. The simplest, low cost and rapid method is the detection of acid-fast bacilli by microscopy. However, 40 to 60% of patients with pulmonary disease and 75% of patients with extra-pulmonary disease are

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smear negative, and in this situation even contemporary and confirmatory culture methods take several weeks to become positive.^{2,3} Therefore, a number of alternative rapid diagnostic tests that use molecular, chromatographic and immunological methods have been developed in different periods of time. While molecular methods overcome the insensitivity of the smear method and the time required for culture, they depend upon retrieval of a specimen from the site of infection. This is often difficult in cases of tuberculosis in children and in some cases of extra-pulmonary disease.⁴

Serological test for diagnosing active Tuberculosis (TB) based on antibody detection IgM, IgG and IgA were commercially available kits for decades, although no international guidelines have recommended their use.⁵ It is estimated that 1.5 million serological tests for tuberculosis, mainly enzyme linked immunosorbent assay (ELISA) were performed in private sector in India.⁵ The estimated cost in Indian currency was around ₹825 million/year (USD 15 million).⁶

Subsequently it was found that these tests are inaccurate and imprecise. According to the survey serological tests are widely used in countries with higher prevalence of tuberculosis cases like Afghanistan, Bangladesh, Brazil, Nigeria, Pakistan, Cambodia, China, India, Kenya, Thailand and Vietnam etc.6 Recently it has been reported the misdiagnosed tuberculosis cases by serological tests. The systemic review was conducted by WHO in special program for research technology in tropical diseases (TDR). It has been found later on that sensitivity and specificity of these tests are highly variable from 1% to 60% and 53% to 99% respectively. The performance of result declined further in HIV infection.

With this present knowledge a study has been conducted in 115 patients who have symptoms suggestive of tuberculosis and Mantoux positive to see the status of ELISA test in urban and rural based undernourished children of low socioeconomic status. In all the cases confirmation was made after giving a course of antituberculosis treatment as par protocol suggested by IAP Pediatric Drug Formulary 2009. The criteria of cure taken as

- Subsidence of all the symptoms that were present prior to start of therapy
- Improvement in sense of wellbeing
- Positive weight gain (1.5-3 kg) during last six months which is more than normal, i,e., accelerated growth. Thus these findings are suggestive of improvement from the disease treated - tubercular infection.

Immunological methods use the specific humoral or cellular responses of the host to infer the presence of infection or disease. They do not require a specimen from the site of infection. Numerous serological tests that use various antigens, such as secreted and heat shock proteins, lipopolysaccharides, and peptides, have been developed. These tests use various modifications of enzyme-linked immunosorbent assay (ELISA) or immune-chromatographic methods to detect different antibody classes.^{4,11} The serological test is an immune response when nutrition of the child is one of the factors. It is not well known about the status of undernourished and low socioeconomic status of MT positive cases in urban and rural areas. Not only the nutrition status, these children are the victims of recurrent respiratory tract infection and diarrheal diseases. Thus a serological study of IgM and IgG have been conducted on 115 children who are Mantoux positive and of nutritional grade 1 to 3 malnutrition according to NCHS scale. We have evaluated ELISA serological tests to determine their performances with sera from four groups of individuals with suspected TB. Study was done before the banning of the serological test by ELISA for TB by WHO in 2011 to see the status in urban and rural areas in low socioeconomic status and undernourished children. 12,13

METHODS

The present study was conducted on 115 children who were suffering from clinically suspected pulmonary tuberculosis (PT) and those who had enlarged cervical lymph nodes as extra pulmonary tuberculosis (EPT).

Inclusion criteria

Group 1

Cough persisting for more than 6 weeks and not responding to two courses of antibiotics: 60 cases

Group 2

Irregular temperature for 6 weeks without any localizing signs and not responding to two courses of antibiotics: 22 cases

Group 3

Cough with documented chest findings either clinically (presence of crackles and wheeze on auscultation) or radio logically (Hilar and /or mediastinal lymph nodes): 18 cases

Group 4

Multiple cervical lymph nodes, non-tender and of size> 1.5 cm or matted: 15 cases

The cross-sectional observational study was conducted from September 2010 to February 2011 at a tertiary care teaching institute, West Bengal, India. Institutional ethics committee permission was taken before starting of study. Informed consent was signed by the parent (s) or in case of young adolescent assent had been taken in addition.

For all the cases MT test was done by a trained person in presence of one of the authors and reading was taken by the same author within 48 to 72 hours. The inoculating material used was PPD - 5 TU. The vial once opened was used within 48 hours and the residua was discarded. All were human immunodeficiency virus negative. A standard dose of five tuberculin units (TU) (0.1ml) was injected intradermally (into the skin) and reading was noted 48 to 72 h later. PPD-RT 23 with tween 80 of strength 1 TU and 2 TU was standardized tuberculins available in India supplied by the bacillus calmette-guérin (BCG) vaccine laboratory, Guindy, Chennai, India.

It is to be injected strictly intradermally, using 28 or 26-gauge needle and tuberculin syringe from which 0.1 ml can be delivered accurately. A discrete, pale elevation of the skin (a wheal) 6 to 10 mm in diameter should be produced when the injection is given correctly. The results of this test must be interpreted carefully. The person's medical risk factors determine the size of induration the result is positive (5 mm, 10 mm, or 15 mm).

ELISA

ELISA using both the kits was performed on blood (serum) obtained from all study cases by both the assay systems.

ERBA ELISA (TB IgG) test

The test was performed to detect IgG antibodies to A60 antigen using commercially available kits (Anda Biologicals, Strausberg; France) according to manufacturer's instructions. Serum dilution of 1:100 was used in the assay. Positive and negative reference sera were included in run along with test sera. For determining

IgG units, the curve was constructed by plotting the OD values of different reference sera. Thereafter, concentration of IgG antibodies in test serum in terms of units/ml were determined by extrapolating the OD value of serum against the reference curve. 15,16

SEVA TB (IgG) ELISA test

Stick indirect penicillinase ELISA was performed as described by Nair et a1, to detect IgG antibodies in human sera. Five µl of optimally diluted antigen (ES-31 (0.2pg/ml), a glycoprotein with metalloprotease activity having N-terminal sequence NTGQS (Asp-Thr-Gly-Glu-Ser) purified by fast protein liquid chromatography from culture filtrate of M. tb H37Ra as described by Nair et al previously, applied to cellulose acetate membrane squares fixed to plastic strip, optimally diluted human sera (1:600) and anti-human IgG penicillinase conjugate (1:1000) were used in this assay.

The sera showing complete decolourisation of blue colour substrate at least 5 min. earlier than the negative control denoted a positive reaction. ¹⁶

RESULTS

Out of 115 cases, malnutrition is graded as 65 had grade 1, 35 cases had grade 2 and 15 cases had grade 3. Socioeconomic status (SES): class 3 (lower middle) 21, class 4 (upper) 30, class 5 (lower) 64.

Mantoux test reading

10 mm - 15 mm: 60 cases
15 mm - 20 mm: 30 cases
20 mm - 30 mm: 25 cases

Table 1: Demographic characteristics of the study participants.

Group	Number	Hindu (%)	Muslim (%)	Male (%)	Female (%)	1-3 years (%)	3-6 years (%)	6-9 years (%)
1	60	40 (66.6%)	20 (33.4%)	32 (53.3%)	28 (46.7%)	20 (33.3%)	22 (36.7%)	18 (30%)
2	22	14 (63.6%)	8 (36.4%)	12 (54.5%)	10 (45.5%)	9 (40.9%)	7 (31.8%)	6 (27.3%)
3	18	10 (55.5%)	8 (44.5%)	10 (55.5%)	8 (44.5%)	6 (33.3%)	7 (38.9%)	5 (27.8%)
4	15	8 (53.3%)	7 (46.7%)	8 (53.3%)	7 (46.7%)	3 (20%)	10 (66.7%)	2 (13.3%)
Total	115	72 (62.6%)	43 (37.4%)	62 (53.9%)	53 (46.1%)	38 (33%)	46 (40%)	31 (27%)

Table 2: Statistical analysis of IgM and IgG positives in the study participants with suspected TB.

Antibody	Positive (percentage)	Negative (percentage)	Total	\mathbf{x}^2	P-value
IgM	51 (44.3%)	64 (55.7%)	115	0.158	< 0.5
IgG	54 (46.9%)	61 (53.1%)	115	0.138	< 0.5

Present study showed that ELISA IgM for TB sensitivity and specificity was 44.3% and 55.7% respectively. Study

results also showed that ELISA IgG for TB sensitivity and specificity was 46.9% and 53.3% respectively.

Both Ig M and Ig G both were found to be positive in 43 (39.4%) cases (Table 2).

Table 3: Comparative sensitivity and specificity of ELISA TB Kit in other study. 13

Kit	Assay technique	Sensitivity	Specificity
Panthozyme TB complex plus	ELISA	37%	100%
Qualisa TB	ELISA	100%	99%
T. Mitra, New Delhi	ELISA	80%	97%
TB TROP dis res centre	ELISA	97%	99%
Span diagnostics	ELISA	94%	97%

DISCUSSION

Following infection children have a higher risk not only of progression to disease, but also of extra-pulmonary dissemination and death. ^{18,19} Infants have a particularly high morbidity and mortality from TB.20 While many factors including host genetics, microbial virulence and underlying conditions that impair immune competence (e.g. malnutrition and HIV infection) determine the outcome of infection, it is likely that the high rate of progressive TB seen in young children is largely a reflection on the immaturity of the immune response. The diagnosis of tuberculosis is very difficult and controversial in children below 6 years of age. Diagnostic difficulties pose the greatest challenge to childhood TB management. TB is often not considered in the differential diagnosis in children, especially in low endemic settings. TB can mimic many common childhood diseases, including pneumonia, generalised bacterial and viral infections, malnutrition and HIV. However the main impediment to the accurate diagnosis of active TB is the pauci-bacillary nature of the disease in children. Younger children also produce smaller amounts of sputum, which is usually swallowed rather than expectorated. Bacteriological samples may be collected by conducting early morning gastric washings, a fairly unpleasant procedure that requires hospital admission and overnight-fast for up to three consecutive nights. Consequently bacteriological confirmation is the exception rather than the rule with only 10-15% of sputum samples revealing acid fast bacilli (AFB) and culture remaining negative in around 70% of cases with probable TB. 21,22

The gold standard test is the isolation of *Mycobacterium tuberculosis* from specimens like sputum, gastric lavage, nasopharyngeal aspirate or tissue specimen by fine needle aspirate. For positivity of AFB, it needs at least 1000 organism per ml of sputum, whereas 10-1000 viable organisms are enough to yield culture positive.²³ But in children, it is very difficult to collect sputum and very difficult to get gastric lavage for consecutive three days.

The positivity of AFB in sputum is 20-40% in very good centers. ²⁴ The sensitivity of sputum for AFB is 50% or less. ^{25,26} X-ray chest is good one when military mottling, consolidation, collapse or pleural effusion is present. It is very difficult to assess lymph node size in thoracic cavity by plain chest X-ray. The suggestive lymph node size is > 1.5 cm, which can be detected by CT chest which is costly and private sector charges around five thousands rupees at present. There are other methodologies like ELISA test which detects antibodies of IgM, IgG and Ig A against tuberculous antigens. There specificity and sensitivity is variable (37% - 100%) and (97-100%).¹³ But in the presents study IgM sensitivity 44.3%, specificity 55.7%; IgG sensitivity 46.9%, specificity 53.3%. This is much lower than the other studies done by different authors. Antibody-profiling in blood or antigendetection in urine has been attempted by many groups, mainly in adult patients. A review of serological tests concluded that commercial antibody detection tests for extrapulmonary TB have no role in clinical care or case detection.27-30

Mx test represents a dermal response to tuberculin antigen-an antibody reaction reflecting the immune response of the individual. Although a weak serological test with low specificity and negative predictive value, it is commonly used as a screening test in developing countries like India. In present study, Mx test was positive in 100 % of the patients who underwent the test. ELISA was done in 115 patientsonly, 39.4 % of whom showed sensitivity. This is a costly test that our patient population could not afford. Another drawback is that ELISA remains positive even after therapy. The response to mycobacteria is variable, and its reproducibility is poor.³¹ Hence, the value of immunological tests remains undefined in clinical practice. The conclusion is that no serological investigation is perfect, and it is unlikely that serological tests alone will provide the diagnosis in all cases.

The newer nuclear technologies like PCR, nucleic acid amplification test, Interferon immunoassay are good but availability of these tests are beyond the reach of parents of children of low socioeconomic status in urban or rural areas. In the present study the serological tests show that it is not significant at all (p - value < 0.5) and the sensitivity and specificity of IgM and IgG are much lower in spite all the patients were symptomatic and with anti-tubercular treatment. The other immunological test is interferon gamma release assay (IGRs). IGRs done by two methods 1) quantiferon TB gold 2) T - spot Test. Recently it has been reported the misdiagnosed TB in this serological tests also. 32,33 Detected pulmonary and extra pulmonary tuberculosis found highly variable in sensitivity and specificity.³⁴ The sensitivity of the serological tests ranged from 1 - 60% and specificity 53 -99%. The test performance declined more in HIV infection. The grades of recommendation, assessment, development and evaluation (GRADE) has done an updated systematic review and recommended against the

currently available serological tests.³⁵ In July 2011 WHO issued a policy statement that commercial tests provide inconsistent and imprecise estimation of sensitivity and specificity. There was no evidence that existing commercial serological assays improve the existing patient - important outcomes and proportion of false positive and false negative results adversely impact patient safety.

Overall data quality was graded as very low with harm/risks far over weighing any potential benefits. It is therefore recommended that these tests should not be used in individuals suspected of active pulmonary or extra pulmonary tuberculosis irrespective of their HIV status.

In Pulmonary tuberculosis, 67 studies were reviewed, including 32 studies from low- and middle-income countries. The results demonstrated that sensitivity and specificity from individual studies were highly variable. Pooled results of the most widely used tests showed sensitivity at 76% and 59% and specificity at 92% and 91% in smear-positive and smear-negative patients respectively. An evaluation by the TDR programme of 19 rapid commercial tests, in comparison with culture plus clinical follow-up, showed similar variability with sensitivity values of 1% to 60% and specificity of 53% to 99%. ¹²

In extrapulmonary tuberculosis, 27 studies were reviewed, including 10 studies from low and middle income countries. The results demonstrated that sensitivity and specificity values from individual studies were highly variable. Pooled sensitivity was 64% for lymph node TB and 46% for pleural tuberculosis. Pooled sensitivity and specificity for the most widely used tests were 81% and 85% respectively. In a single study involving HIV-infected patients, the sensitivity of the test was 33%. 12

Low sensitivity results in an unacceptably high number of patients being wrongly given the 'all clear' (i.e. a falsenegative). This can lead to them dying from untreated tuberculosis, and the disease also being transmitted to others. Low specificity leads to an unacceptably high number of patients being wrongly diagnosed with TB (i.e. a false-positive). This can lead to them undergoing a six month course of unnecessary treatment, while the real cause of their illness remains un-investigated and undiagnosed immediately following the WHO policy, the RNTCP published against the use of serological tests in India. 12,36

This is the first 'negative' policy recommendation on TB issued by WHO and was developed in compliance with the GRADE process for evidence synthesis and formulation of recommendations. It was approved by the WHO guidelines review committee having satisfied the requirements for guideline development and issued in July 2011. Recently Indian Academy of Pediatrics

discouraged the serological tests and other medical associations like Indian Association of Medical Microbiologists, Indian Chest Society and Indian Medical Association followed the suit.³⁷ In India's vast private sector, until recently serological (blood) tests were used to diagnose TB even though they have no clinical basis for diagnosing TB. Yet, more than 73 types of serology kits for TB diagnosis are being marketed and produced, mostly in China and India. The market in India for these tests was estimated at a \$15 million a year. In 2012, India issued a notification banning the manufacture, sale and distribution of serology-based testing kits.

India continues to report more than two millions tuberculosis cases every year and undiagnosed and misdiagnosed tuberculosis is partly responsible for that.³⁸ To overcome the problem Government of India has set up an ambitious goal to provide universal quality diagnosis and treatment for all tuberculosis patients in the country by national strategic plan 2012-2017.

CONCLUSION

The ELISA serological test of antibodies has false positivity and negativity. This leads over diagnosis or under diagnosis of tuberculosis. The ELISA has been done by different authors who have done all categories patients irrespective of their nutritional status. SES have much higher value in specificity and sensitivity than the present study. In the present study all are undernourished low SES. Antibodies levels depend of the immunological response. The nutrition is one the factor for immunological response. Thus antibody level by ELISA in malnourished less informative than well-nourished children. There is no evidence that existing commercial serological assays improve patient outcomes, and high proportions of false-positive and false-negative results may have an adverse impact on the health of patients

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Institutional Ethics Committee

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