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Laboratory Investigations on the Diagnosis of Tuberculosis in the Malnourished Tribal Population of Melghat, India

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Abstract

Background

Malnutrition is a major risk factor for the development of tuberculosis (TB). In India, Melghat is among the tribal regions which consist of highest number of malnutrition cases. Because of the paucity of TB data from these malnourished areas there is an urgent need for the development and evaluation of improved TB diagnostic tests. In the present study, three in house developed diagnostic tests namely TB-Ag(antigen) ELISA, Adenosine deaminase (ADA) estimation and IS*6110* polymerase chain reaction (PCR) assay were investigated for the detection of *Mycobacterium tuberculosis* (*M. tb.*) infection.

Methods

For investigation, blood samples were collected from 128 study subjects from six villages of Melghat tribal area and evaluated using three in house developed assays, namely TB-Ag ELISA, ADA estimation and IS*6110* PCR.

Results

The TB-Ag ELISA method yielded 83% sensitivity and 94% specificity. The ADA and PCR assay gave a sensitivity of 61% and 49% and specificity of 62% and 98% respectively. A considerable good agreement of 82.81% (k=0.472) between TB-Ag ELISA and PCR was observed. The overall sensitivity

of TB-Ag ELISA was significantly higher ($p<0.05$) than the ADA and PCR while PCR yielded highest specificity among all the three evaluated tests.

Conclusions

We concluded that the routine use of TB-Ag ELISA can be useful for screening of suspected TB patients in the malnourished population where sophisticated laboratory set up is difficult.

Introduction

Tuberculosis (TB) is a major health problem worldwide. As per the World Health Organization (WHO) report of 2010, India is the highest TB burden country with an estimated TB incidence figure of 2.3 million cases out of a global 9.4 million cases. The WHO statistics also showed that India is 17th out of the 22 high burden countries in terms of TB incidence rate [1]. It is estimated that about 40-50% of the Indian population is infected with TB bacteria, the vast majority of whom have latent rather than active TB. The association between TB and malnutrition is well recognized; TB can lead to malnutrition and malnutrition may predispose to TB [2]. TB has been found to coexist with malnutrition among patients at the time of starting treatment in both developed and developing countries [3]. TB is also associated with various socioeconomic factors and often occurs in populations suffering from poverty, poor housing and economic deprivation and these are also the major factors predisposing to poor nutritional status and impaired immune function [4].

Melghat is a hilly forest tribal area in Maharashtra State of India with a tribal population of nearly 0.28 million. Nearly 80% of the population of this region suffers from malnutrition. Most of the villagers (>90%) are farmers or labourers, with below poverty line status (>75%) and living in kaccha houses without electricity (>90%) and having high illiteracy rates (>50%). In Melghat, our investigation team has observed that TB is one of the most important and leading cause of death in the economically productive age group of 16-40 years. However, to the best of our knowledge, data of TB incidence have not been yet documented in this tribal region.

Earlier in our laboratory, we have developed ELISA based TB antigen (Ag) detection test based on the evaluation of Ag 85 complex in the body fluid of TB patients [5,6]. Adenosine deaminase (ADA) has also been extensively evaluated in the body fluids of pulmonary TB (PTB) and extra pulmonary TB (EPTB) patients with reliable sensitivity and specificity [7]. Both the developed methods are inexpensive, relatively simple, and can be performed in any pathology laboratory where sophisticated tests are not available. In addition to that, an in house *IS6110* based Polymerase chain reaction (PCR) assay was also developed in our laboratory to detect *Mycobacterium tuberculosis* (*M. tb.*) DNA in cerebrospinal fluid (CSF) samples of Tuberculous meningitis (TBM) patients [8]. Because of the paucity of TB data from this malnourished area, there is a need for evaluating TB diagnostic tests for the rapid diagnosis of the patients in such remote region. The objective of the present study is to evaluate the performance of above mentioned three tests TB-Ag ELISA, ADA and PCR in the malnourished tribal population to estimate the burden of TB in Melghat region.

Materials and Methods

Ethics statement

The study was approved by Ethical Committee of Central India Institute of Medical Sciences (CIIMS), Nagpur and Meditation, Aids, Health Addiction and nutrition (MAHAN) Trust, Melghat. Written consents were taken from each participant after detailed oral explanation about the study. Since the study was also conducted on children, a written informed consent was obtained from the immediate caretaker, or next of kin, prior to inclusion, on behalf of children participating in the study.

Study design

The present study was conducted in high burden malnutrition tribal region of Melghat villages, Amravati district, India. The region has high prevalence of undiagnosed TB along with other infectious diseases. The participants recruited were mostly tribal's form six different villages of Melghat most of

which had poor infrastructure development and socioeconomic status, no provisions of electricity, toiletries and ate nutrients deficient diets. Each participant was asked to complete a questionnaire about his/her possible risk of exposure to *M. tb*. Details of any prior Tuberculin skin test (TST), presence of underlying illnesses, infections experienced in the last 3 months, medication, history of previous TB or anti-TB treatment and daily dietary intake were also recorded. Baseline characteristics such as age, gender, weight, height, type and duration of exposure with active TB patients, occupation, and education were recorded. Bacillus Calmette–Guérin (BCG) vaccination status was assessed based on examination of BCG scar on left forearm.

Samples

Blood samples (n=128) were collected in plain tube and on FTA card for TB-Ag ELISA, ADA test and for **the** DNA isolation followed by PCR amplification of *M. tb*. specific gene *IS6110*. **The** collected samples were stored at -20°C or at room temperature until further analysis.

Subjects

Study populations were classified into following two categories on the basis of their clinical **characteristics.**

***ActiveTBsubjects* (*n=41*): TB (n=37) was confirmed if acid fast bacilli (AFB) and/or culture of sputum specimens were positive for *M.tb*. When both tests were negative (n=04), the patients were diagnosed based on clinical symptoms**. Clinical suspicion of TB was based on minimum of 3 of the following symptoms a) Chronic cough with or without expectoration/hemoptysis/chest pain of more than 2–3 weeks or past history of TB b) Fever more than 2–3 weeks c) Progressive unexplained weight loss d) loss of appetite e) night sweats f) or by chest radiographs g) response to anti TB treatment (ATT).

***Non-TBcontrols* (*n=87*)**: This category included participants from the same villages. All the control cases included in this group were TST, sputum and culture negative with no clinical, bacteriological features of TB, and normal chest radiograph with no history of ATT.

TB Antigen ELISA test

M. tb. specific antigen (Ag85) in the serum samples were evaluated by the indirect ELISA protocol as described earlier by Kashyap et al. [5]. Negative reference control was selected from the pooled serum of non-TB healthy controls that had never been exposed to TB, and the absence of Ag 85 complex antigen was demonstrated by immunoblotting with specific rabbit antibodies against Ag 85 complex antigen [6]. Positive reference control Ag85 complex from M. *tb* strain H $_{37}R_{v}$ was obtained from Colorado State University, USA through TB Research Materials and Vaccine Testing Contract (NO1-AI-40091) [5]. A single dilution (10µg/ml) of the positive stock was made in the negative reference serum to achieve a defined reactivity, which was within the accurate detection limits of the ELISA. ELISA was run with the positive serum control, negative serum control and sample blank (PBS). Three replicates of positive and negative controls were included on each ELISA plate along with the test sample. A sample with an absorbance of >0.18 was considered positive for ELISA result as previously reported [6]. The accuracy of test was evaluated by determining its sensitivity and specificity as compared to reference standards**.**

ADA

ADA activity in serum samples was determined by the method of Guisti and Galanti [9] based on the Berthlot reaction, which is the formation of coloured indophenol complex from ammonia liberated during deamination of adenosine. The development of colour was quantified by spectrophotometer (Systronic, India). One unit of ADA is defined as the amount of enzyme required to release 1mMol of ammonia/min from adenosine at standard assay conditions. Results were expressed as units per liter per minute (U/L/min). A sample with cut-off value of ≥15 U/L/min was considered as a positive test result.

DNA isolation

Sample collection and DNA isolation was done by FTA Elute Technology. 100 µl of whole blood samples obtained by venous puncture were applied to FTA Elute matrix. The samples were dried thoroughly and kept at room temperature until use. For isolating the DNA, a 3 mm disc was punched from the cards containing the samples and placed in a microcentrifuge tube. The punch was rinsed in 500 µl water and pulse vortexed three times for 5 seconds. Water was removed and tube was centrifuged for 5 seconds. 30µl sterile water was added to it, sample was heated at 95°C for 60 min; pulse vortexed 30 times; and centrifuged at 12000 rpm for 10 min. 10 µl of eluted DNA was used further for PCR.

IS6110 gene PCR

Identification of *M. tb*. was done using a specific pair of primers designed to amplify an insertion sequence IS*6110* in the *M. tb*. complex with the expected band size of about123-bp. The sequence of these primers are: *5′ CCT GCG AGC GTA GGC GTC GG 3′* and *5′ CTC GTC CAG CGC CGC TTC GG 3′* respectively. A 50 µl reaction contained 10× assay buffer (Applied Biosystems, USA), 10 mM dNTP's (Applied Biosystems, USA), 10 pmole of each primer (Sigma Aldrich, USA), 2.5 units Taq DNA Polymerase (Applied Biosystems, USA) and 5 µl of extracted DNA. Amplification was carried out in a thermal minicycler (peqlab Biotechnologie GmbH, Erlangen, Germany), which involved 40 cycles of denaturation at 94°C for 1 min, annealing of primers at 68°C for 1 min, and primer extension at 72°C for 1 min. In each independent PCR assay, test results were compared with the results for one positive and one negative control. The positive controls included the DNA of H_{37}Rv strain provided by Colorado State University, USA, (Contract No 1-A1-40091). No-template control reaction consisted of sterile water instead of target template. The amplification products were separated on 2% agarose gels, visualized on a UV- light transilluminator (Biotech R & D Laboratories, Yercaud, Salem, India) and photographed. The test samples were analyzed on the basis of presence or absence of 123 bp product as compared to the positive control DNA.

Statistical analysis

Statistical analysis was performed using the MedCalc statistical software (version 10.0.1). Optimal cut-off value for the ADA