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Research Article

DETECTION OF MYCOBACTERIAL ANTIBODIES IN SERUM SAMPLES USING BCG VACCINE BASED ELISA

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ABSTRACT

Tuberculosis (TB) is an infectious disease that is causing considerable morbidity and mortality. Improved diagnostic methods are needed for the detection of *Mycobacterium tuberculosis* infections, and the development of a sero-diagnostic test would complement presently available diagnostic methods. Enzyme-Linked Immunosorbent Assay (ELISA) for diagnosis of tuberculosis has been widely explored over the years. The purpose of this study is to reduce the time duration required for the diagnosis of tuberculosis. Antibody response was studied using ELISA against antigens extracted from BCG. 30 samples were analysed for its specificity and sensitivity. The optimal cut-off point chosen was at 1 of the reactivity of the high positive control serum sample (PC). In that 20 samples were positive and the sensitivity and specificity of the test appeared to be 90.9%. The high sensitivities, specificities and promising antigenic combination in detection of TB suggest their potential application in diagnosis of TB.

Keywords: ELISA, BCG, Mycobacterial antigens, Tuberculosis

INTRODUCTION

Approximately one-third of the world's population is infected with *Mycobacterium tuberculosis*, and 7 to 8 million new cases of tuberculosis (TB) occur each year[1]. It is a major socio- economic burden in India, affecting 14 million people, mostly in the reproductive age group (15-45 years). It is involved in about 5-16% of cases of infertility among Indian women[2-4], though the actual incidence may be under reported due to asymptomatic presentation of genital tuberculosis (GTB) and paucity of investigations.

The majority of TB cases occur in developing countries with limited resources and the diagnosis of TB largely depends upon clinical examination and radiographic findings, confirmed by sputum smear microscopy as well as bacterial culture. Culture is not useful as a first line of diagnosis due to the long cultivation period required for *M. tuberculosis* (6 to 8 weeks); hence, confirmation of the diagnosis relies on sputum smear examinations. However, smear microscopy has a sensitivity of only 50 to 60%, and the detection rate in children and patients co-infected with human immunodeficiency virus (HIV) is even lower[5]. Accurate and early diagnosis of tuberculosis is crucial for effective patient management and TB control. Moreover, accurate identification of TB is the key to preventive of the disease among persons at risk.

Despite the enormous global burden due to TB and overall low rates of case detection, conventional approaches to diagnosis continue to rely on test that have major drawbacks. Smear microscopy lacks sensitivity and cases of infectious tuberculosis are missed[6], therefore isolation of *M. tuberculosis* through sputum culture either on solid or selective liquid medium remains the gold standard in TB diagnosis[7]. This technique increases sensitivity as well as specificity but requires several weeks before the results are known. The main problem is among the patients of extrapulmonary tuberculosis and children, who characteristically do not produce sputum. Moreover, the yield of tubercle bacilli in these patients is poor[8-12]. Extra pulmonary tuberculosis remains an important diagnostic and therapeutic problem[13].

The purpose of this study is to reduce the time duration required for the diagnosis of the organism *Mycobacterium tuberculosis*. Even though the gold standard diagnostic tool for the organism is culture, the time duration for incubation masks its specificity. In order to overcome this, a variety of diagnostic tools can be developed that have sensitivity rate of culture techniques. We have extracted antigens from the organism and used it for the diagnosis of infection by ELISA technique.

MATERIALS AND METHODS

Serum samples

Serum samples from untreated bacteriologically confirmed sputum positive and sputum negative TB cases were used in this study. The serum samples from tuberculosis patients and healthy individuals with no history of TB were obtained from Microbiological Laboratory, Coimbatore, India. The serum samples were confirmed for its positivity by inoculating on to modified Middlebrook 7H9 broth with PANTA (polymyxin B, amphotericin B, nalidixic acid, trimethoprim and azlocillin) cocktail of antibiotics and incubated for 1 week at 37 °C. Magenta pink colored granular deposition was indication of growth. They were also confirmed by Zeihl-Neelsen method of acid fast microscopy. The samples showing positivity in the culture were considered as positive and the samples that did not show any growth were considered as negative. Serum samples were stored at –20 °C after adding sodium azide (0.1%) for preservation.

Extraction of BCG antigens

BCG strain was obtained commercially (Tubervac, Serum Institute of India) as freeze-dried BCG strain, which is usually used for vaccination. The strain was reconstituted by adding 0.5 mL of sterile saline solution and rolled for proper mixing. The organism was inoculated onto thyroxine supplemented Lowenstein-Jensen slants (Hi-Media, Mumbai) and incubated at 37 °C for 6 weeks[14]. After incubation bacilli were inactivated in 5 mL of 5% phenol for 1 hr at 4 °C. The cells were washed twice in normal saline and then suspended in 4 mL of 0.05 mol/L phosphate-buffered saline (PBS), pH 7.2. The bacilli were sonicated with 30-s bursts (15%, 150W) at 1 min intervals for 30 min at 4 °C. The sonicate was incubated with 2 mL of sodium dodecyl sulfate (SDS) extraction buffer (5% SDS, 5% 2-mercaptoethanol, and 8 mol/L urea in 0.01 mol/L PBS, pH 7.2) in boiling water bath for 5 min, followed by incubation at 4 °C for 24 hrs, spin the incubated sample at 10,000 rpm at 4 °C for 30 min, the supernatant was separated, dialyzed against 0.01 mol/L PBS; pH 7.2; for 48 hrs. The protein content of the antigen was determined by Lowry *et al.* method [15]. The antigen was stored at -20 °C till future use[16].

Coating of ELISA plates

The ELISA plates were coated as per the procedure described by Kadival *et al.* with some modifications[17]. Two 96 welled U bottomed polystyrene microtitre plates (Tarsons, India) were coated with the antigens (50µg/mL in PBS). 50µL of this solution was transferred into the wells of ELISA (Microtitre plates). The plate was kept in the refrigerator for overnight. The next day plate was washed with PBS tween solution for 3 times. 5mL of 20x PBS solution was taken and its volume was made into 1x solution with distilled water. To this 100mL of solution 2g of skimmed milk powder was added. The plated were decanted after overnight incubation and non specific binding sites were blocked by adding 150µL of 1% PBST milk was added into antigen coated ELISA plate. Then the plate was incubated at 37 °C for 2 hours. The plates were blot dried and were stored in the refrigerator.

Cut-off determination

The cut-off value separating positive from negative sera was calculated as 3 standard deviations above the mean of the OD values obtained from negative samples.

ELISA assay

The ELISA was performed in 96 welled polystyrene microtitre plates as per the procedure described by Chaturvedi *et al.* with some modifications[18]. Briefly, 1% PBST milk was prepared (0.5g of skimmed milk powder was added to 50mL of PBST solution). 400µL of PBST milk was added into each dilution tube. 100µL of sample was added to it. These 1:5 dilutions were mixed well. The microtitre plates were marked as BCG IgG and BCG IgM. The first well is the blank and to the second well positive control was added. From the dilution tubes 50µL was transferred to the ELISA plate wells in duplicates and plates were incubated at 37 °C for 1hr and 30 min. After incubation, the plates were washed for six times with PBST solution and it were blot dried. The conjugate IgG HRP and IgM HRP (Sigma, USA) of 5µL were added to 15 mL and 5mL of PBST milk (diluents). 50µL of IgG conjugate was added to the plate (1:3000 dilution) BCG IgG and 50µL of IgM conjugate was added to plate (1:1000 dilution) BCG IgM. The plates were then incubated at 37 °C for 1 hr. After the incubation, the plates were washed with the wash buffer (PBST) for 8 times and were blot dried. 75µL of the substrate (ortho phenlyene diamine dihydrochloride in phosphate citrate buffer and hydrogen peroxide) were added into each well of ELISA plates. The plates were then kept in a dark place for 30 minutes for colour development. The colour change was noted and the reaction was stopped using the stop solution 1N sulphuric acid (50µL) into each well of ELISA microtitre plates. The plates were read using the ELISA reader at 492 nm.

RESULTS AND DISCUSSION

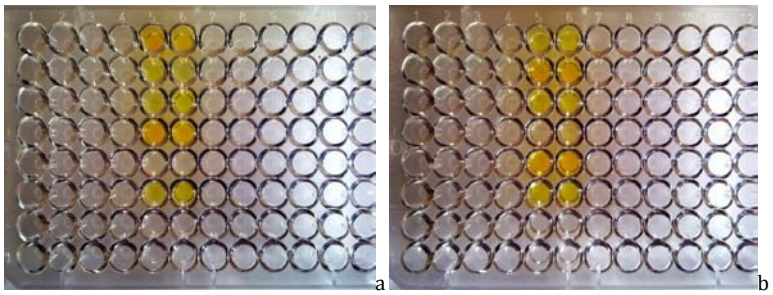
Extra pulmonary TB is often difficult to diagnose because of its diverse clinical presentations[19, 20]. Tuberculosis is a disease of great antiquity and has probably caused more suffering and death than any other bacterial infection[21]. Early diagnosis of this infection is of 'utmost concern' for successful control[22]. Moreover, the sensitivity of antibody detection tests is much lower in HIV seropositive patients co-infected with tuberculosis[23].

The ELISA cut-off point was determined from 8 negative samples and it was calculated as 1. Results lower than 0.9 is considered as negative and those greater than 1.1 considered as positive results. Those results between 0.9-1.1 are considered as suspected results and should be re-evaluated with fresh samples after a while.

Thirty clinical samples were analyzed out of which 20 appeared to be positive. Among the positive samples, 8 samples showed high IgG positivity; 6 samples showed high IgM positivity and remaining 6 samples showed both IgG and IgM positivity. IgG positivity showed chronic state of infection and IgM positivity showed acute state of infection. All samples were inoculated onto LJ slants for confirmation of infection. In the LJ culture 22 cases appeared to be positive. Sensitivity was calculated as follows:

$$\frac{\text{Total number of positive in ELISA}}{\text{Total number of positive cases in culture}}$$

Earlier studies demonstrated detectable anti-TB antibody in CSF[24-26]. The detection of antibodies correlated well with disease and was positive in 68-80% of cases. Several authors have shown that antigen or antibody detection is useful in the diagnosis of pulmonary[27], meningeal, pleural and abdominal tuberculosis by radioimmunoassay[8, 24, 28]. In a limited study of 10 CSF samples from patients with tuberculous meningitis, Sada *et al.* (1984) showed the potential usefulness of ELISA in the detection of antigen[29].



a b

Fig. 1: Microtitre plates showing IgG (a) and IgM (b) response. The color of the substrate (golden yellow) indicates positivity of the samples

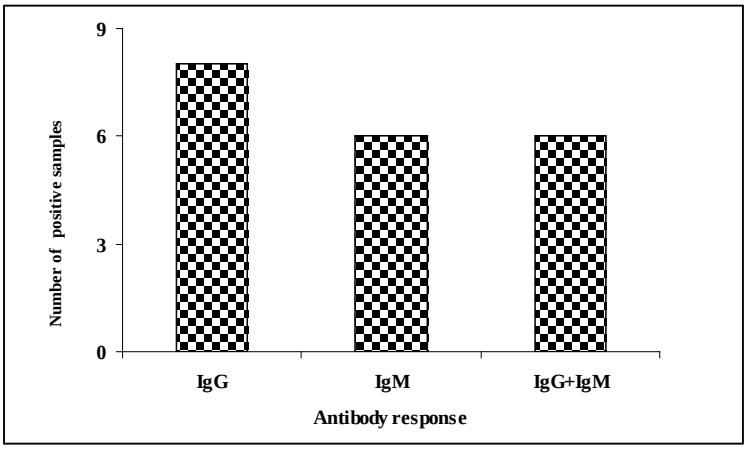


Fig. 2: Antibody response

CONCLUSION

In this study, we have developed a specific and sensitive assay for the detection of anti-TB antibody, which can be used for the diagnosis of tuberculosis. This can be adopted as a method of choice for the diagnosis of mycobacterial infections in cases where suspicion is high, in combination with other clinical criteria and could be an alternative to other more expensive sophisticated techniques.

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