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Research Article

IMMUNODIAGNOSIS OF TUBERCULOSIS USING PCR AND BCG VACCINE BASED ELISA TECHNIQUE

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ABSTRACT

Tuberculosis causes immense consequences on society. One person out of three is infected with *M. tuberculosis* and accounts for about 2 billion worldwide. Diagnosis of tuberculosis (TB) remains an enigma despite many technological developments. The main purpose of this study is to develop a diagnostic tool which can yield results within short period of time and much higher sensitivity rate. In this study, 200 serum samples were used for developing ELISA by using BCG strain as source of diagnostic antigen. Out of 200 assayed serum samples, 149 samples gave positive results with the sensitivity rate of 88.7%.

Keywords: BCG, tuberculosis (TB), sonicate antigen, culture filtrate antigen, ELISA, PCR.

INTRODUCTION

Tuberculosis (TB) caused by *M. tuberculosis* is the second leading cause of death among all infected individuals and is responsible for 2 billion deaths annually [1]. TB accounts about 2.5 % of the global burden of disease and is the commonest cause of death in young women, killing more women than all causes of maternal mortality combined. TB holds seventh place in the global ranking of causes of death currently. The disease primarily infects people in developing countries, with the most endemic regions being Africa, South East Asia and the Western Pacific. Clinical differentiation of tuberculosis and other mycobacteriosis is difficult due to overlapping domain of symptoms [2].

The immunological methods commonly studied for diagnosis of tuberculosis are based on detecting serum antibodies against mycobacterial antigens, which can be proteins, lipids, or polysaccharides. Few commercial tests based on the detection of specific antigens, such as the 38 kDa protein, have been developed and have been in use, primarily in developing countries [3]. Tests based on detection of antibodies against *M. tuberculosis* are important alternative to traditional methods for diagnosis of active tuberculosis because they can detect immune response induced during infection. This response is either not present or reduced in asymptomatic infected subjects.

To increase the sensitivity and specificity attainable with pure antigens, it has been proposed to use a mixture of different immunodominant antigens to cover the whole range of possible responses by all individuals in a population. With this technique, a sensitivity rate of 94 % was achieved [4, 5].

Enzyme-linked immunosorbent assays (ELISA) are sensitive and reproducible and do not require sophisticated equipments. Antibody-based tests for TB diagnosis have been developed for more than 2 decades. They are attractive because of their technical simplicity, rapidity, and low cost. In addition, they permit to avoid the microscopic examination step, which may be difficult to achieve in poorly equipped hospital laboratories. The present study was, undertaken to standardize a rapid, sensitive and inexpensive antigen detection ELISA for the diagnosis of tuberculosis.

Materials and Methods

Serum samples

200 serum samples from patients with TB were used in this study. The serum samples were obtained from neighbouring diagnostic laboratories as well as hospitals. 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.

Strain used for antibody detection

BCG Strain

BCG strain was obtained commercially (Tubervac, Serum Institute of India) as freeze-dried BCG strain, which is usually used for vaccination.

Polymerase Chain Reaction

All serum samples were confirmed for the presence of IS*6110* gene which is present in positive samples only by polymerase chain reaction.

DNA extraction

The DNA was extracted by CTAB (Cetyl Trimethyl Ammonium Bromide)-phenol chloroform extraction method. 0.2 ml of clinical samples was centrifuged at 10,000 rpm for 10 min. The supernatant was discarded and the pellet suspended in 567 µL of TE buffer (Tris EDTA, pH 7.4), 30 µL 10% SDS and 3 µL proteinase K (20 mg/ml), mixed and incubated at 37 °C for 1 h. After incubation, 100 µL of 5 M NaCl and 80 µL of high-salt CTAB buffer (containing 4 M NaCl, 1.8% CTAB (cetyl-trimethyl- ammonium bromide) was added and mixed followed by incubation at 65 °C for 10 min. An approximate equal volume (0.7–0.8 µL) of chloroform-isoamyl alcohol (24:1) was added, mixed thoroughly and centrifuged for 5 min in a micro-centrifuge at 12,000 rpm. The aqueous viscous supernatant was carefully decanted and transferred to a new tube. An equal volume of phenol: chloroform- isoamyl alcohol (1:1) was added followed by a 5 min spin at 12,000 rpm. The supernatant was separated and then mixed with 0.6 volume of isopropanol to get a precipitate. The precipitated nucleic acids were washed with 75% ethanol, dried and re-suspended in 100 µL of TE buffer. DNA was extracted from clinical samples and from BCG strain. Each step of the extraction protocol was performed inside biosafety cabinet, using protected tips and dedicated pipettes at room temperature.

PCR amplification of DNA

Two set of primers genus specific and species specific were used for the assay.

The sequences of the genus specific primers were:

Forward primer: 5' GAGATCGAGCTGGAGGATCC 3',

Reverse primer: 5' AGCTGCAGCCCAAACCTGTT 3' amplified a 383 base pair fragment of a gene that codes for a 65 KDa protein present in all species of mycobacteria.

The sequences of the species specific primers were

Forward primer: 5' CCTGCGAGCGTAGGCGTCGG 3',

Reverse primer: 5' CTCGTCCAGCGCCGCTTCGG 3' amplified a 123 base pair nucleotide sequence in IS*6110* present in strains of the *M. tuberculosis* complex (*M. tuberculosis, M. bovis, M. africanum, M. microti*). The primers were reconstituted and stored in 100 µM and 5 µM stock solutions at – 20 °C.

Amplification of DNA

DNA amplification by PCR was performed with a total reaction volume of 25 µL by using model Eppendorf Thermo Cycler. Contents were well mixed and subjected to thermocycling as follows:

- For mycobacterium genus specific reaction, the conditions were: The conditions were, initial denaturation at 94 °C for 5 min followed by 30 cycles of denaturation at 94 °C for 90 sec, annealing at 57 °C for 90 sec, extension at 72 °C for 90 sec and final extension at 72 °C for 5 min.
- For *M. tuberculosis* complex, the conditions were: The conditions were, initial denaturation at 94 °C for 5 min followed by 30 cycles of denaturation at 94 °C for 2 min, annealing at 68 °C for 2 min, extension at 72 °C for 2 min and final extension at 72 °C for 5 min.

PCR products were detected on 1.5% agarose gel in 1X TAE buffer containing ethidium bromide at 10µg/ ml concentration under ultra violet illumination. When the single band of 383 bp and 123 bp were obtained, it was inferred that the sample was positive for Mycobacterium species and *M. tuberculosis complex* respectively.

Antigen extraction

Extraction of BCG sonicate antigens

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 and incubated at 37 °C for 6 weeks. After incubation bacilli were inactivated in 5 mL of 10% formaline 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 sec bursts (15 %, 150 W) 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. After centrifugation 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's method [6]. The antigen was stored at -20 °C till future use [7]. This antigen was marked as BCGSE.

BCG culture filtrate antigen preparation.

BCG culture filtrate antigen was prepared as described earlier [7]. BCG was cultured in synthetic Sauton medium enriched with 0.5% glucose, 0.5% sodium pyruvate and 0.05% tween 80 for 3 weeks (35 °C; 5%-10% CO2). The culture was autoclaved at 121 °C for 60 min and was centrifuged at 12,000 rpm for 30 min at 4 °C; the supernatant fluid was sterilized by filtration through syringe filter using a 0.22 µm-pore-size membrane. The proteins were precipitated with 80% ammonium sulfate in cold overnight, dissolved in sterile phosphate-buffered saline (PBS) and dialyzed at 4 °C until free of ammonium ions. The protein content was determined by the method of Lowry against a bovine serum albumin (BSA) standard [6] and adjusted to 5mg/ml. The protease inhibitor phenylmethylsulfonyl fluoride was added to a concentration of 10 mM. The culture filtrate preparations were stored at -20 °C until required. This antigen was marked as BCGCFA.

Antibody detection ELISA assay using BCGSE antigen

Coating of ELISA plates

The ELISA plates were coated as described earlier [8]. Two 96 welled U bottomed polystyrene microtitre plates were coated with the antigens (50µg/mL in PBS). 50µL of this solution was transferred into the wells of ELISA. The plate was kept in the refrigerator for overnight. The next day plate was washed with PBST 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 plates were decanted after overnight incubation and non specific binding sites were blocked by adding 150µL of 1% PBST milk 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.

ELISA assay

The ELISA was performed in 96 welled polystyrene microtitre plates [8]. 1% PBST Milk was prepared (0.5g of skimmed milk powder was added to 50mL of PBST solution. This is the sample diluent). 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 BCGSE IgG and BCGSE 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 minutes. After incubation, the plates were washed for six times with PBST solution and it were blot dried. The conjugate IgG HRP and IgM 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) BCGSE IgG and 50µL of IgM conjugate was added to plate (1:1000 dilution) BCGSE 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.

Antibody detection ELISA assay using BCGCFA antigen

Coating of ELISA plates

The ELISA plates were coated as described earlier [8]. Two 96 welled U bottomed polystyrene microtitre plates were coated with the antigens (50µg/mL in carbonate bicarbonate buffer). 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 PBST 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 plates were decanted after overnight incubation and non specific binding sites were blocked by adding 150µL of 1% PBST milk into antigen coated ELISA plate. Then the plates were incubated at 37 °C for 2 hours. The plates were blot dried and were stored in the refrigerator.

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were mixed well. The microtitre plates were marked as BCGCFA IgG and BCGCFA IgM. The first well was 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 minutes. 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) BCGCFA IgG and 50μL of IgM conjugate was added to plate (1:1000 dilution) BCGCFA 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 (tetramethyl benzidine tetrahydrochloride in 0.1 M phosphate citrate buffer and 0.01% 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 450 nm.

Cut-off determination

The ELISA cut-off point was determined as per the kit manufacturer's guidance. Briefly, cut-off point was determined from 8 negative samples and it was calculated as 1. Results with lower optical density (OD) than 0.9 is considered as negative and OD 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.

Validation of ELISA assay

For validation of test, all serum samples were tested with commercially available ELISA kit for IgG and IgM antibody detection. The procedure was followed as per the kit manufacturer's guidance. The antigen coated ELISA plates were kept in room temperature 30 min prior the commencement of test. 10 X concentrated sample dilution buffer was made to 1 X (1 mL of sample dilution buffer mixed with 9 mL of distilled water) concentration. The serum sample (200 μL) was mixed with 500 μL of 1 X sample dilution buffer. The diluted sample (50 μL) was added to IgG and IgM ELISA plates and kept in incubator for 1 h and 30 min at 37 °C. After incubation, the plates were washed with washing buffer for six times provided in the kit and blot dried. IgG and IgM HRP labelled conjugates were diluted with conjugate buffer and 50 μL of the conjugates were added to IgG and IgM ELISA plates respectively. The plates were incubated at 37 °C for 1 h and washed again with washing buffer (PBST) for four times and blot dried. Chromogen substrate (75 μL) was added to both plates and incubated in dark at room temperature for 30 min. After incubation, the reaction was stopped by adding 50 μL of stop solution (1 N sulphuric acid) into each well of ELISA microtitre plates. The plates were read using ELISA reader at 492 nm.

Calculation of sensitivity of ELISA assay

The sensitivity of the ELISA assay was determined by using the following formula.

$$\text{Sensitivity} = \frac{\text{No. of positive cases in ELISA}}{\text{No. of positive cases in commercially available ELISA kit}}$$

Results and Discussion

In this study, 200 clinical samples were analyzed out of which 149 appeared to be positive. Antigen BCGSE: Among the positive samples, 69 samples showed high IgG positivity; 49 samples showed high IgM positivity and remaining 31 samples showed both IgG and IgM positivity. Antigen BCGCFA: Among the positive samples, 68 samples showed high IgG positivity; 47 samples showed high IgM positivity and remaining 34 samples showed both IgG and IgM positivity. IgG positivity showed chronic state of infection and IgM ositivity showed acute state of infection. By using commercially available ELISA kit, 168 samples appeared to be positive out of 200 serum samples. 80 samples showed high IgG positivity; 54 samples showed high IgM positivity; and remaining 34 samples showed both IgG and IgM positivity.

The use of *M. tuberculosis*-specific antigen cocktail in ELISA provides an easy, cheap, and effective alternative to the molecular methods for the development of a specific test for TB in endemic countries. In the present study, the total number of cases in ELISA appeared to be 149. Total number of cases in commercially available kit appeared to be 168. Hence, the sensitivity of the test appeared to be 88.7 %. The diagnostic value of a given test in clinical practice depends on its positive and negative predictive values [9]. These values vary markedly with the prevalence of the disease in a given community [10]. The high positive predictive values of tests would make a positive test result useful in strengthening the clinical suspicion, but a negative result would be less useful [11]. Moreover, the predictive values of positivity in high endemic areas may be achieved by increasing the specificity, i.e. by modifying the cut off point separating positive from negative test result. However, increased specificity can only be gained at the expense of the sensitivity [12].

Early diagnosis of this infection is of 'utmost concern' for successful control. Although sonicate and purified antigens isolated from *M. tuberculosis* have been extensively explored in diagnosis, it will be of considerable interest to identify a circulating mycobacterial antigen in blood, which will be a better marker for confirming active infection. Several authors previously reported good results in antibody detection using different preparation, including complex antigens [13, 14].

Various reports have been published with wide variations in methodology employed by other workers on dot-ELISA for detection of mycobacterial antigen in variety of clinical samples (CSF, pleural fluid, sputum, etc.) and yielding good results. Reported sensitivity for antigen detection ranged from 78 - 89 % and specificity of 73 - 100 % in sputum specimens [15, 16], while it was found to be 72 - 89 % and 87 - 98 %, respectively in CSF samples of patients suffering from tubercular meningitis [17, 18].

Scientists made a comparative study of the immunoglobulin G (IgG), IgM and IgA antibody responses to four trehalose containing glycolipids purified from *M. tuberculosis*; diacyltrehaloses, triacyltrehaloses, cord factor and sulfolipid I (SL-I) [19]. The results indicated a significantly elevated IgG and IgA antibody response in tuberculosis patients, compared with controls, with all the antigens used. SL-I was the best antigen studied, showing test sensitivities and specificities for IgG of 81 and 77.6 %, respectively, and of 66 and 87.5 % for IgA. Using this antigen and combining IgA and IgG antibody detection, high test specificity was achieved (93.7 %) with a sensitivity of 67.5 %.

Studies have reported that the present ELISA appears to be highly sensitive and specific and could detect as little as 5 ng of protein/ mL [20]. They found that the test was 91.6 % sensitive and 85.7 % specific. Only 8.3 % of false-negativity was observed which might be due to the level of antigen below the detectable limit in these specimens, or could be the manifestation due to an antigen-antibody immune complex-mediated reaction.

Similar results have also been obtained in other studies using purified or crude antigen of *M. tuberculosis* [21] and *M. habana* TMC 5135 [22].

Earlier studies have used TB 72 in serodiagnosis [23]. As this antibody reacts to *M. bovis*, serodiagnosis with this antibody in a BCG vaccinated population needs to be fully explored. Antigen detection [24, 25] after affinity purification may be more useful in diagnosis than antibody, as a high antigen concentration indicates current infection.

In addition, changes in antigen conformation that may occur as a result of passive coating of the antigens to solid supports may cause technical artifacts resulting in false positive and false negative reactions [26]. On the other hand, many serological assays have been developed for specific antibody detection in TB patients [11, 12, 14].

Enzyme immunoassays use either the direct or indirect methods [27, 28, 29]. In the direct method, polyclonal anti-G antibodies as capture reagent, and the same antibodies linked to an enzyme as conjugate were used for detecting the reaction. These features allowed greater specificity in the antigen-antibody reaction and also simplify the test procedure. It is possible that optimization of the assay changes with each lot of antibody production. However, the standard conditions for the ELISA test can be maintained by titrating each new polyclonal antibody lot, which can be achieved by determining the optimal antibody concentration which allows the correct discrimination between a positive and a negative sample.

CONCLUSION

Developing nations pose a major threat against tuberculosis. As its control relies on early detection of cases and effective treatment, timely diagnosis of TB is essential ensuring a favourable clinical outcome. Research for detection of a suitable antigen for serodiagnosis of tuberculosis has come a long way and yet there is lot more to be explored.

In this study, sera from 200 TB patients were evaluated for the presence of antibodies against sonicated extract (SE) and culture filtrate (CFA) antigens. By using BCGSE ELISA assay, 69 samples showed high IgG positivity; 49 samples showed high IgM positivity and remaining 31 samples showed both IgG and IgM positivity. By using BCGCFA ELISA assay, 68 samples showed high IgG positivity; 47 samples showed high IgM positivity and remaining 34 samples showed both IgG and IgM positivity. By using commercially available ELISA kit, 168 samples appeared to be positive out of 200 serum samples. 80 samples showed high IgG positivity; 54 samples showed high IgM positivity; and remaining 34 samples showed both IgG and IgM positivity. The sensitivity of the test appeared to be 88.7 %. This methodology does not require any sophisticated equipment or special skilfulness and offers the opportunity of investigating a large series of samples in a short period.

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