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Rapid Immunodiagnosis of Tuberculosis by using Polymerase Chain Reaction and in-house developed Enzyme linked Immunosorbent Assay

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Abstract

Tuberculosis (TB) remains the second leading cause of death among all infected individuals and is responsible for 2 million deaths annually. The definitive diagnosis of TB is by demonstrating acid-fast bacilli either by direct smear or culture. Since these methods are insensitive (smear technique) and time consuming (culture technique), additional immunodiagnostic methods such as antigen or antibody demonstration has become necessary. The main purpose of this study is to develop a diagnostic tool which can yield results in short span of time with much higher sensitivity rate. In this study, 200 serum samples were used for the development of ELISA technique. Out of 200 samples, 149 gave positive results and the sensitivity of the developed test appeared to be 88.7%.

Keywords: $H_{37}Ra$, tuberculosis (TB), antigen extraction, ELISA, PCR.

Introduction

Tuberculosis is caused by *Mycobacterium tuberculosis*. Diagnosis of tuberculosis (TB) remains an enigma despite many technological developments. Techniques which are sensitive and specific to detect *M. tuberculosis* in clinical specimens are important for the diagnosis of tuberculosis. The disease primarily infects people in developing countries, with the most endemic regions being Africa, South East Asia and Western Pacific. Differentiation of tuberculosis and other mycobacterial infection is difficult due to similar symptoms[1].

Enzyme linked immunosorbent assay (ELISA) has been used with increased sensitivity for detecting the antibodies. Most of the antibody response in tuberculosis is directed towards those antigens common to all mycobacteria and to some other genera. In TB patients, the serological response to mycobacterial antigens has been primarily evaluated using standard ELISA with in-house methodologies. In ELISA, *M. tuberculosis* antigens are adsorbed onto a solid phase where they capture specific mycobacterial antibodies from serum samples. Antibody capture is detected with a second antibody conjugated to an enzyme (often peroxidase), which reacts with its substrate (hydrogen peroxide in the case of peroxidase) so indicating when antibody-antibody reaction has taken place[2].

A sensitivity of 90 % was achieved with a cocktail of 9 antigens like ESAT6, 14 kDa, MPT63, 19 kDa, MPT64, MPT51, MTC28, 30 kDa, 38 kDa, and KatG[3]. One of the advantages of using protein antigens for immunodiagnosis is that they can be prepared using recombinant DNA technology. With this recombinant antigen, a sensitivity of 94 % was achieved[4, 5].

ELISA is preferred because they are rapid, technically simple and low in cost. In addition, they avoid the microscopic examination, which may be difficult to achieve in poorly equipped diagnostic laboratories. The present situation demands for specific, sensitive, rapid, simple, safe and cost-effective diagnostic tests. The present study explores the potential of detecting mycobacterial antibodies in serum samples.

Material 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: ATCC Strain: *M. tuberculosis* $H_{37}Ra$ (ATCC 25177 purchased from MicroBioLogics, USA) was used.

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[6] 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 *M. tuberculosis* standard strain H_{37}Ra. 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 *M. tuberculosis* sonicate antigens: *M. tuberculosis* H_{37}Ra 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 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. 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[7]. The antigen was stored at -20°C till future use[8]. This antigen was marked as MTSE.

***M. tuberculosis* culture filtrate antigen preparation:** *M. tuberculosis* H_{37}Ra culture filtrate antigen was prepared as described earlier[9]. *M. tuberculosis* H_{37}Ra was cultured in synthetic Sauton's medium enriched with 0.5% glucose, 0.5% sodium pyruvate and 0.05% tween 80 for 3 weeks (35°C; 5%-10% CO_2). The culture was autoclaved for 121°C for 60 min and was centrifuged at 10,000 rpm for 45 min at 4°C, and 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 MTCFA.

Antibody detection ELISA assay using MTSE 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 (Microtitre plates). The plate was kept in the refrigerator for overnight. The next day plate was washed with PBS tween (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.

ELISA assay: The ELISA was performed in 96 welled polystyrene microtitre plates as described earlier[8]. Briefly, 1% PBST Milk was prepared (0.5g of skimmed milk powder was added to 50mL of PBST solution. This is the sample diluent). 500µL of PBST milk was added into each dilution tube. 100µL of sample was added to it. These 1:6 dilutions were mixed well. The microtitre plates were marked as MTSE IgG and MTSE IgM. The first well was blank and to the second well positive control serum was added. From the dilution tubes 50µL was transferred to the ELISA plate wells in duplicates and plates were incubated at 37°C for 2hrs. After incubation, the plates were washed for four times with PBST solution and were blot dried. The conjugate IgG HRP and IgM HRP 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) MTSE IgG and 50µL of IgM conjugate was added to plate (1:1000 dilution) MTSE IgM. The plates were then incubated at 37°C for 2 hrs. After the incubation the plates were washed with the wash buffer (PBST) for six 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 1 N 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 MTCFA antigen: Coating of ELISA plates: The ELISA plates were coated as described earlier[9]. 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 PBS tween (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.

ELISA assay: The ELISA was performed in 96 welled polystyrene microtitre plates as described earlier[9]. Briefly, 1% PBST Milk was prepared (0.5g of skimmed milk powder was added to 50mL of PBST solution. This is the sample diluent). 300µL of PBST milk was added into each dilution tube. 100µL of sample was added to it. These 1:4 dilutions were mixed well. The microtitre plates were marked as MTCFA IgG and MTCFA 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. After incubation, the plates were washed for six times with PBST solution and it were blot dried. The conjugate IgG HRP and IgM HRP 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) MTCFA IgG and 50µL of IgM conjugate was added to plate (1:1000 dilution) MTCFA 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 1 N 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 MTSE: Among the positive samples, 70 samples showed high IgG positivity; 48 samples showed high IgM positivity and remaining 31 samples showed both IgG and IgM positivity. Antigen MTCFA: Among the positive samples, 65 samples showed high IgG positivity; 49 samples showed high IgM positivity and remaining 35 samples showed both IgG and IgM positivity. IgG positivity showed chronic state of infection and IgM positivity 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 specific test for diagnosis of 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 %. ELISA technique for *M. tuberculosis* antigen detection is easy to perform, in-expensive and allows simultaneous processing of multiple samples. However, the ELISA assay developed in this study will help to improvise the diagnosis of tuberculosis.

There are facts that should be recognized for the serological diagnosis of tuberculosis. The cell wall antigenic composition of tubercle bacilli differs among various isolates[10]. The patient does not develop protective antibodies against all antigenic determinants in the cell walls of bacilli and the specificities of the developed antibodies differ among individuals.

Earlier studies have confirmed that sandwich ELISAs can be used to detect small amounts of *M. tuberculosis* antigens in body fluids[11]. In order to optimize the detection of mycobacterial antigens in pleural and ascitic fluids and CSF, these fluids were used in serial dilutions. Optimal dilutions for the detection of mycobacterial antigens were thus defined as 1:50 (pleural and ascitic fluids) and 1:5 (CSF). Using these dilutions, ELISA demonstrated high specificity and sensitivity, with no false negative readings being recorded.

Experts studied that out of 68 smear-positive TB cases, using cocktail antibody, a sensitivity rate of 97% (66/68) for immune-complexed cocktail antigen and 91% (62/68) for free cocktail-antigen detection was observed, compared to 91% (62/68) for immune-complexed ES-31 and 79% (54/68) for free ES-31 antigen when anti–ES-31 antibody was used alone[12]. Thus, combination of antibodies showed improved sensitivity and was better than single antibody. The specificity was observed to be 99% for immune-complexed antigen using cocktail antibody. The analysis of different groups of TB patients showed circulating immune complexed antigen to be a sensitive marker than free antigen.

M. tuberculosis bacilli, their lipid extracts and secreted proteins are major targets of specific immunity in TB. Previous data suggests that no single antigen is suitable for reliable serodiagnosis of tuberculosis[13]. One explanation might be that different stages of the disease may give antibody responses to different antigens. Also both glycolipid and protein antigens have been found essential to account for the total load of antibodies in a patient's body so as to provide acceptable sensitivity (proteins) and specificity (glycolipids)[14,15]. The combination of specific antigens like ESAT-6 and CFP-10 proved to be highly sensitive and specific for both in-vivo and in-vitro diagnosis. In humans, the combination had a high sensitivity rate (73 %) and a much higher specificity rate (93 %) than purified protein derivative (7 %)[16].

Studies have showed the potential usefulness of ELISA in the detection of antigen[17]. The discrepancy in findings between the present and an earlier study[18-23] can be explained by the difference in the number of patients and difference based on the detection of antigenic fractions. The test was found to be appropriate as it is rapid, simple and easy to perform. In addition, it does not require any sophisticated instruments or special skill and helps in investigating a large number of samples in a short span of time.

Conclusion

Presently, the diagnosis of TB depends on clinical findings and various laboratory tests. Although several commercial diagnostic assays like Geno-Type Mycobacterium CM/AS are available to identify various species of mycobacteria as they cannot be used on large numbers of samples due to their high cost. Therefore, immunodiagnosis is ideally suited as a diagnostic method when cost matters. Serological diagnostic tests like ELISA can be preferred because of their cost effective performance in laboratories.

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 MTSE ELISA assay, 70 samples showed high IgG positivity; 48 samples showed high IgM positivity and remaining 31 samples showed both IgG and IgM positivity. By using MTCFA ELISA assay, 65 samples

showed high IgG positivity; 49 samples showed high IgM positivity and remaining 35 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 %. The detailed methodology provided here will enable different laboratories to standardize and carry out this test routinely.

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