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Research Article

SEROLOGICAL DIAGNOSIS OF MYCOBACTERIAL INFECTION USING ANTIGEN DETECTION BASED IN-HOUSE DEVELOPED IMMUNOBLOT TECHNIQUE

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ABSTRACT

Objective: Tuberculosis is one of the major health concerns in developing countries. Early diagnosis of tuberculosis is essential for effective start of chemotherapy. The higher mortality rate of tuberculosis is due to lack of rapid diagnostic tool which is cost effective as well as lack of sophisticated methodology that can lead to prompt diagnosis in time. There are several methods available to diagnose the infection presently in the market but the question arises about its specificity and sensitivity.

Methods: Immunoblot technique was developed based on nitrocellulose paper by using capture and detection antibodies raised in mice and rabbits against sonicated extracts (CSP) of *Mycobacterium tuberculosis*.

Results: As tuberculosis generates a highly heterogeneous antibody repertoire, its diagnosis requires tests based on antigens. A new, rapid immunoblot assay for antigen-based diagnosis, which can detect mycobacterial antigens in serum specimens, was developed. Antigen detection of 200 sera samples from clinically suspected patients by immunoblot assay gave a sensitivity rate of 96.3 % and specificity rate of 70.8 % by using Bayesian analysis.

Conclusion: The results of the assay proved to be superior to conventional methods and combined with clinical data, could form the basis for starting an earlier course of treatment. This immunoblot assay can simplify the identification of *M. tuberculosis* in clinical laboratories.

Keywords: Tuberculosis, $H_{37}Ra$, Antigens, Immunoblot, Polyclonal antibodies, Bayesian analysis.

INTRODUCTION

Tuberculosis has been major cause of morbidity and mortality around the world. It is estimated that nearly 1 % of the world population is newly infected each year and that approximately one-third of the world population is latently infected with *Mycobacterium tuberculosis*.

Specific mycobacterial antigens can be identified in patients using ex vivo generated monoclonal antibodies. The tests based on the detection of mycobacterial antigens rather than antibody response to them is likely to prove more successful. A number of antigen-capture assays have been described usually based on enzyme linked immunosorbent assays (ELISA) or agglutination of antibody coated latex particles. ELISA with anti BCG antibody has been successfully used by several workers. They are suitable for detection of antigens in 'clean' materials such as CSF, pleural or peritoneal fluid.

The ability to produce and exploit monoclonal antibodies (mAbs) has revolutionized many areas of biological sciences. The unique property of a mAbs is that it is a single species of immunoglobulin (IG) molecule. This means that the specificity of the interaction of the paratopes on the IG, with the epitopes on an antigenic target is the same on every molecule. This property can be used to great benefit in immunoassays to provide tests of defined specificity and sensitivity; which improve the possibilities of standardization. There is an inverse relationship between antigen and antibody so that estimation of both greatly increased the diagnostic efficiency [1, 2, 3, 4].

MATERIALS AND METHODS

Serum samples

Two hundred serum samples were collected from patients suspected with TB, were obtained from various diagnostic laboratories as well hospitals were used in this study. 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. 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. Blood sample was procured from clinical suspected patients with tuberculosis. After collection, blood was transferred to penicillin bottle and kept in incubator at 37 °C, to make blood clot. After 30 min of incubation, the blood sample was centrifuged for 15 min at 5,000 rpm to separate serum from clot. Serum samples were stored at –20 °C after adding sodium azide (0.1 %) for preservation.

ATCC Strain *M. tuberculosis* $H_{37}Ra$ (ATCC 25177 supplied from MicroBioLogics, USA) was used for preparation of antibodies.

Extraction of *M. tuberculosis* antigens

M. tuberculosis was inoculated onto thyroxine supplemented Lowenstein-Jensen slants and incubated at 37 °C for 6 weeks [5]. 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 sec bursts (15 %, 150 W) at 1 min intervals for 30 min at 4 ºC. 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 g 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].

Preparation of antibodies

The ethical clearance for using animal models regarding this study has been obtained from the institutional ethical clearance committee. The animals were handled and taken care off as per the guidelines provided by the ethical clearance committee.

Capture antibody: Capture antibody was raised in Swiss albino mice in two batches of two each; against sonicate extract of *M. tuberculosis*. All mice were male, aged between five and six weeks and weighing between 40 and 50 g. Twenty seven micrograms of extract was mixed with an equal volume of Freund's complete adjuvant (Sigma) and injected subcutaneously. After 2 weeks, a booster dose of extract mixed with Freund's incomplete adjuvant (Sigma) was given. Finally, after two weeks, blood was collected from the immunized mice; pooled serum was separated and stored at -20 °C without any preservative [8].

Detection antibody: Detection antibody was raised in one batch of two male adult New Zealand rabbits against extract of *M. tuberculosis*. In the primary immunization, 2 ml of water in oil emulsion that contained 270 µg of extract was mixed thoroughly with incomplete Freund's adjuvant (Sigma). The material was injected in divided doses into two gluteal intramuscular sites and two flank subcutaneous sites. The immunization schedule was repeated on the 14th, 21st, 28th and 35th days following the primary immunization. Finally, after two weeks, blood was drawn from the ear veins; pooled serum was separated and stored in small aliquots at -20 ºC without any preservative [8].

Anti rabbit antibody: Goat anti rabbit antibody was obtained commercially. This antibody was enzymatically labelled by the method prescribed by Hudson and Hay [9]. 2 mg of horseradish peroxidase (HRPO) was dissolved in 0.1 ml (0.2 M) solution of sodium metaperiodate, and the volume was brought up to 0.6 ml with distilled water. The mixture was stirred gently for 20 min at room temperature. The mixture was dialyzed against 0.001 M sodium acetate buffer overnight at 4 ºC. To this 0.2 ml of sodium carbonate buffer (0.2 M) was added (pH 9.5) followed by addition of 1 ml anti rabbit antibody to be conjugated. This solution was vigorously mixed in a shaker for 2 hrs at room temperature. 50 µl of freshly prepared sodium borohydride solution was added to the mixture. The mixture was further dialyzed overnight against 0.1 M borate buffer, pH 7.4 at 4 ºC, with 4 changes. One millilitre of sodium azide solution (stabilizer) was added to the conjugate and stored at -20 ºC [10, 11].

Purification of antibodies

The antibodies were purified by immunoabsorbent affinity column chromatography [12]. Briefly, 1 g of cyanogen bromide-Sepharose 4B (Sigma) was reconstituted to 3.5 ml in distilled water and was washed with large volumes (20 times the original gel volume) of cold 0.1 M sodium bicarbonate buffer (pH 9). This was then resuspended as slurry of 50 % (wt/vol) by the addition of 0.1 M sodium bicarbonate buffer. Sonicate extract of *M. tuberculosis* was added in an equal volume to the activated cyanogen bromide-Sepharose 4B, and the immunoabsorbent was incubated for 16 hrs at 4 °C. The immunoabsorbent was washed five times with large volumes of 0.1 M sodium borate buffer (pH 9) alternating with 0.1 M sodium acetate buffer (pH 5), suspended in 0.1 M phosphate buffered saline (PBS), poured into a glass chromatographic column (diameter 1 cm; length 10 cm), and equilibrated with 0.15 M PBS. The column was washed three times with 4 M urea in 0.15 M sodium bicarbonate buffer (pH 9), alternating with 0.15 M PBS to minimize the leaching out of antigen from the immunoabsorbent column. One millilitre of capture antibody of *M. tuberculosis* $H_{37}Ra$ was added, and the column was run with 0.15 M PBS. Every 10 min, a 1 ml fraction was collected until a blank reading at 280 nm was obtained. The specific mycobacterial antibody that bound to the immunoabsorbent column was eluted with 4 M urea in 0.15 M sodium bicarbonate buffer, and the absorbences of fractions at 280 nm were recorded. Fractions with absorbances of >0.05 were pooled and dialyzed against PBS. The protein content of the dialyzed material was estimated by the method of Lowry [6]; it was stored at -20 °C. The detection antibody was also purified similarly and stored at -20 °C.

Enzyme immunoblot assay

2 µl of purified capture antibody was spotted on strips of nitrocellulose paper (NCP) (0.45 nm pore size) at definite intervals and air-dried at room temperature. Nonspecific sites of sensitized NCP strips were blocked by 3 % bovine serum albumin (BSA) in Tris-buffered saline, pH 7.4, for 2 hrs at 37 ºC. NCP strips were washed in phosphate-buffered saline Tween 20 (PBS-1x; 0.05 % Tween 20, pH 7.2) by gently agitating the solution at 5 min intervals for three times. After the final wash, excess PBS-T was soaked on filter paper. 2 µl aliquot of clinical sample was then applied on spotted circles of first antibody on NCP and allowed to react for 1 hr at 37 °C in a humid chamber. After three washings with PBS-T, NCP strips were exposed with purified detection antibody and incubated for 1 hr at 37 °C. After washing with PBS-T, strips were dipped in 1:500 dilution of anti-rabbit horseradish peroxidase conjugate and allowed to react for 1 h at 37 °C. Unbounded label was then removed by washing the strips with several changes of PBS-T over a 1 hr period. Finally, reaction strips were developed with freshly prepared DAB-substrate solution (3, 3′-diaminobenzidine, 0.6 mg/ml with 6 µl H_2O_2). Reaction was performed in a dark chamber. After 1 min, reaction stopped by washing the NCP with distilled water. Development of brown spot was considered as an indication of positive reaction [8].

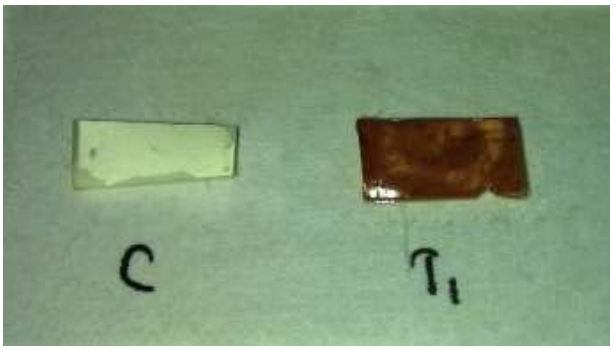
Calculation of sensitivity of immunoblot assay

The sensitivity and specificity of the immunoblot assay was calculated by using Bayesian analysis [13].

RESULTS AND DISCUSSION

Many studies have been done on mycobacterial antigens and their use as diagnostic reagents and/or vaccines for human tuberculosis. Cross referencing of antigens reported in such studies in the past was rather difficult due to variations in the characterization techniques used by various groups. With the advent of polyclonal antibody technology and the greater ease of DNA and protein sequencing, comparisons are more feasible [14].

In the present study, the antibody titres of capture and detection antibodies were estimated to be 1:512. Two hundred serum samples were analysed for detection of infection on the basis of antigen demonstration in the serum samples. This study established polyclonal antibodies, as a fast, sensitive, specific, immunodiagnostic assay for detection of TB patients. Out of 200 samples, 130 samples gave positivity for the infection (Fig. 1).



C- Control **T_1- Test**

Fig. 1: Enzyme immunoblot assay

Demonstration of intact bacilli or breakdown products in the form of antigens has far greater utility. Many workers have found enzyme-linked immunosorbent assay (ELISA) to be a rapid and sensitive technique for the detection of mycobacterial antigen [15, 16, 17]. A simple form of ELISA; dot-ELISA has been found to be very sensitive and rapid for diagnosing pulmonary [15, 18], as well as extrapulmonary tuberculosis, i.e. tubercular meningitis [17].

Even after the development of highly specific immunodiagnostic assay, their widespread clinical use is hampered by non-availability of immunologic reagents that relatively are simpler to prepare and couldn't be available in ample amounts. Affinity chromatographic procedures have been used by several workers to obtain semi purified or purified antigens from the complex antigenic mixtures [19].

Earlier studies reported that use of ELISA and immunoblot as the two methods of screening for specificity revealed interesting aspects [20]. ELISA tended to show the antibodies to be more specific than immunoblot. HGT 1 was shown to be negative with M. bovis BCG, *M. kansasii*, and *M. vaccae* by ELISA. Immunoblots, however, recognized weak bands from *M. bovis* BCG and *M. kansasii*. Similar results were observed with HGT 3a, HGT 4, and HGT 6. The negative values in ELISA may arise because of two factors. One is the arbitrarily chosen cut off values which make it difficult to differentiate between weak and negative reactions. The other reason could be that all the antigens might not bind the plate template equally and thus may give false-negative results.

Immunoblots, on the other hand, have many advantages. In addition to higher sensitivity than ELISA, under the conditions of SDS-PAGE the antigens are unmasked, thus making the epitopes more easily available for detection. The reasons for visualizing multiple bands with HGT 1, HGT 2, and HGT 4 could be that the epitopes recognized by these mAbs are present on a large number of antigenic molecules or that the antigens might be fragmented during sonication and are seen as multiple bands.

Studies were conducted for a prospective study for demonstrating Ag 85 complex in sera from TB patients using a monoclonal antibody (mAb) against the complex [21]. The data demonstrated that, the positivity for Ag 85 complex antigen in cases of confirmed and clinically diagnosed TB patients were 96 % (23/ 24) and 79 % (82/ 104) respectively, while the positivity for the patients in the non-tuberculosis group was 14 % (10/ 69). Overall, the indirect ELISA method yielded 82 % sensitivity and 86 % specificity for diagnosis of TB using the mAb against Ag 85 complex.

This present study outlines the use of a rabbit model to profile the humoral immune response of a panel of strain-specific mycobacterial antigens. The varying responses, particularly in the first few weeks following inoculation, further support the use of antigen cocktails in diagnostics.

Calculation of sensitivity of the test by Bayesian analysis

Bayesian analysis was done to estimate sensitivity and specificity of the test [8, 13]. Bayesian analysis is a statistical procedure which endeavors to estimate parameters of an underlying distribution based on the observed distribution. Bayesian inference is a method of statistical inference in which Baye's theorem is used to calculate how the degree of belief in a proposition changes due to evidence. Bayesian inference is justified by the philosophy of Bayesian probability, which asserts that degrees of belief may be represented by probabilities, and that Baye's' theorem provides the rational update given the evidence [22].

In the present study, immunoblot assay appears to be highly sensitive and specific. Total number of cases in immunoblot assay appeared to be 130. It was found that the test was 96.3 % sensitive and 70.8 % specific by using Bayesian analysis. Similar studies reported that the sensitivity (91.6 %) and specificity (85.7 %) for mycobacterial antigen detection (Table 1) using immunoblot assay by applying Bayesian analysis [8].

Table 1: Application of Bayesian analysis for calculation of sensitivity and specificity

Culture-positive, Blot-positive (A)	Culture-negative, Blot-positive (B)	Culture-positive, Blot-negative (C)	Culture-negative, Blot-negative (D)
130	19	5	46

Sensitivity= [A/(A+C)] X 100= 96.3 %
Specificity= [D/(B+D)] X 100= 70.8 %
n = 200

This test provides a good positive predictive value of 96.3 % which is very useful for diagnosis of symptomatic patients suspected of having tuberculosis. The diagnostic values of this test depend on the context of its use. While a negative result would be useful in excluding disease in a population with a low prevalence of tuberculosis, a positive result could potentially aid in clinical decision making when used with sputa from a group of selected symptomatic patients when there is moderate to high degree of clinical suspicion of tuberculosis [23].

CONCLUSION

The *M. tuberculosis* bacteria infects nearly 2 billion people worldwide and 9 million people each year, while killing greater than 1.5 million people annually. Tuberculosis (TB) causes immense consequences on society. 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.

The aim of this study is to develop sonicated antigens for development of immunoblot assay technique to detect *M. tuberculosis* antigens in serum samples using rabbit and mice anti *M. tuberculosis* antibodies from *M. tuberculosis* $H_{37}Ra$ (ATCC 25177) strain. 200 serum samples were analysed for the diagnosis of infection by immuno blot technique. Out of 200 samples, 130 samples gave positivity for the infection. Bayesian analysis was used for calculation of sensitivity and specificity of the test. It was found that the test was 96.3 % sensitive and 70.8 % specific by using Bayesian analysis. In the present study, immunoblot assay appears to be highly sensitive and specific.

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