

A DOT ENZYME IMMUNOASSAY FOR DETECTION OF IgM ANTIBODIES AGAINST PHENOLIC GLYCOLIPID-I IN SERA FROM LEPROSY PATIENTS

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ABSTRACT : A visual dipstick dot enzyme immunoassay (EIA) for diagnosis of leprosy is described. The assay is based on detection of IgM antibodies against phenolic glycolipid (PGL-I) in sera from leprosy patients. The antigen (PGL-I or synthetic disaccharide of PGL-I) was dotted on a nitrocellulose pad stuck on a plastic strip (dipstick). Sera were used at a dilution of 1:200. Peroxidase coupled mouse anti-human IgM monoclonal antibodies were used as the conjugate. A positive test gave a blue dot against a white background. The test was highly specific for leprosy, and was quite sensitive for detection of bacilliferous (BL/LL) leprosy. The antigen dotted and preblocked dipsticks stored at room temperature upto 4 months of observation period, were unable in the assay.

INTRODUCTION

A visual color test which can put in evidence patients or asymptomatic carriers with high bacillary load would be of utility in leprosy control programme. Such, individuals can be deemed to be potential transmitters of infection to others in the community and chemotherapy of such cases with bactericidal drugs can help in containment of the foyer of infection. A number of immunodiagnostic methods are under development, which include FLA-ABS test (Abe et al, 1976), radiometric assays (Harboe et al, 1978; Sinha et al, 1983), and enzyme immunoassays (Brett et al, 1983; Cho

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et al, 1983; Young and Buchanan, 1983; Ralhan et al, 1985). Previous studies using enzymeimmunoassays (Brett et al, 1983; Cho et al. 1983; Ralhan et al 1985) have indicated the presence of IgM class of antibodies in multi-bacillary (BL/LL) patients against phenolic glycolipid (PGL-I) specific to *M. leprae*. Most of these assays, except one recently reported (Young et al, 1985), demand the use of microtitre plates which are not easily available in leprosy control centres in the country.

We describe here a dipstick visual assay where the colour can serve as a qualitative test for positive cases. In this immunodot assay, either PGL-I extracted from armadillo derived *M. leprae*, or synthetic terminal disaccharide linked to bovine serum albumin (Fujiwara et al, 1984) can be used as an antigen. The test is highly specific for leprosy and patients with active pulmonary tuberculosis or persons living in endemic areas who are exposed to a variety of environmental mycobacteria, do not show positivity in the test.

MATERIAL AND METHODS

Materials

Phenolic glycolipid-I was obtained from Dr. P.J. Brennan, Deptt. of Microbiology, Colorado State University, Colorado, and the WHO IMMLEP. Synthetic disaccharide (ND-BSA) was provided by Dr. Delphi Chatterjee, Deptt. of Microbiology, Colorado State University, Colorado. Sera of patients with leprosy or pulmonary tuberculosis were from Safdarjung Hospital, New Delhi and Tuberculosis Research Centre, Madras. Microtitre plates (Titertek) were from Flow laboratories, USA. Dipsticks with two pads of nitrocellulose membrane (1 cm X 0.5 cm ; 0.45 μ pore size) stuck on to a plastic strip (7 cm x 0.5 cm) were made according to our design by Advanced Microdevices, Ambala, India. Bovine serum albumin (fraction V), tween-20, 4-Chloro-1-naphthol and o-phenylenediamine, were purchased from Sigma, USA. Horseradish peroxidase (HRPO) coupled rabbit anti-mouse immunoglobulins were a product of Dakopatts, Germany.

Methods

Preparation of antibody-enzyme conjugate : Mouse anti-human IgM monoclonal antibodies were prepared from the ascitic fluid of mice injected with HB-57 hybridomas obtained from A.T.C.C. Immunoglobulins were precipitated with equal volume of saturated ammonium sulphate and dialysed against phosphate buffered saline (PBS; 0.05M, PH 7.4, 150mM NaCl). The antibodies were conjugated with Horse radish peroxidase using one-step glutaraldehyde method (Avrameas, 1969).

Preparation of antigen solution:-

(i) *Phenolic glycolipids-I* : 1 mg of PGL-I dissolved in 5ml of carbonate-bicarbonate buffer, 0.2M, pH 9.6, was sonicated just before use. It was diluted in the same buffer to give a final concentration of 200 µg/ml for dipstick dot EIA.

(ii) *Synthetic disaccharide (ND-BSA)* : 3, 6, di-o-methyl glucose-2, 3-di-o-methyl rhamnose linked to BSA. 0.5 ml of lyophilized material containing 50 µg sugar (as glucose equivalent) and 200 µg of BSA was reconstituted in 0.5 ml of distilled water. It was diluted in carbonate-bicarbonate buffer, pH 9.6 to a final concentration of 100 ng/ml for plate EIA, and 20 µg/ml for dipstick dot EIA.

Coating of dipstick : The dipstick was rinsed with trisbuffered saline TBS; 50 mM Tris, 150 mM NaCl, pH 7.4) and dried at room temperature. 200 ng of PGL-I or 20 ng of ND-BSA, contained in 1 µl of carbonate-bicarbonate buffer, pH 9.6, was applied in the centre of the lower piece of nitrocellulose membrane and dried at room temperature. The upper pad of nitrocellulose membrane served as internal control. Non-specific binding sites were blocked with 3% BSA dissolved in TBS for 1 hr at 37°C.

Procedure for dot EIA : The antigen coated preblocked dipstick was immersed in patient's serum diluted 1 :200 in TBS-BSA (1%) and incubated at 37°C for 1 hr. After three washings with TBS, the dipstick was kept for 1 hr at 37°C with 2ml of peroxidase coupled mouse anti-human IgM monoclonal antibodies diluted 1 : 500 in TBS-BSA (1%). The dipstick was washed four times and immersed in substrate solution. Substrate was prepared by dissolving 4-chloro-1-naphthol (3mg/ml) in anhydrous methanol diluting 1:5 with tris buffer, and adding (1 µl/ml) 30% H₂O₂. A positive test was indicated by a blue spot developed within 5 to 10 min against a white background.

Procedure for microtitre plate EIA : Plate EIA with ND-BSA was performed according to the method described elsewhere (Ralhan et al, 1985) with some modifications. ND-BSA was used at a concentration of 10ng/well and phosphate buffered saline containing 0.1% tween-20 (PBST) was used. IgM antibodies against ND-BSA were titrated in the assay.

RESULTS

Figure I gives the typical results obtained with the dipstick assay. Sera from lepromatous leprosy (LL) patients gave a clearly visible. blue coloured

dot differentiable from the internal reagent control on the upper pad in the same dipstick. Sera from patients with active pulmonary tuberculosis were negative in the assay. As antigen, both PGL-I and ND-BSA were usable. However, ND-BSA gave equal or slightly better colour with the same serum.

A total of 223 sera were analysed by the immunodot and microtitre plate assays. These included 173 sera from leprosy patients of different categories, 20 normal healthy controls from Delhi, 10 healthy controls from endemic area (Madras), and 20 sera from patients with active pulmonary tuberculosis. In each case, the assay was performed with both PGL-I and ND-BSA. The intensity of the colour in the immunodot assay varied from faint blue to deep blue (Figure 2) which correlated in most cases with the absorbance in microtitre plate EIA. The dot assay was usually not distinct for samples giving absorbance below 0.160 for ND-BSA. Figure 3 gives data on absorbance of all the sera analysed in microtitre plate EIA using ND-BSA as the antigen.

Shelf life and stability

Dipsticks dotted with 'antigen and blocked with BSA as described in methods, were kept at room temperature (20-30°C) and at 4°C and analysed at 3 weeks interval. Table 1 gives the result on a pooled LL serum, using the dipsticks stored under the above mentioned conditions. Dipsticks stored at room temperature upto 112 days of observation period were usable in the assay without any significant deterioration in the readability.

TABLE 1 : Shelf life and stability of antigen coated dipsticks.

DAYS STORED	4°C	ROOM TEMPERATURE (20-30°C)
21	+ + +	+ + +
42	+ + +	+ + +
63	+ + +	+ + +
84	+ + +	+ + +
112	+ + +	+ + +

DISCUSSION

Assay for IgM antibodies against the phenolic glycolipid or terminal sugars have both advantages and limitations. Being a component unique to *M. leprae*, the assay is highly specific and discriminates fully between LL and tuberculosis patients. The antibodies if present against environmental

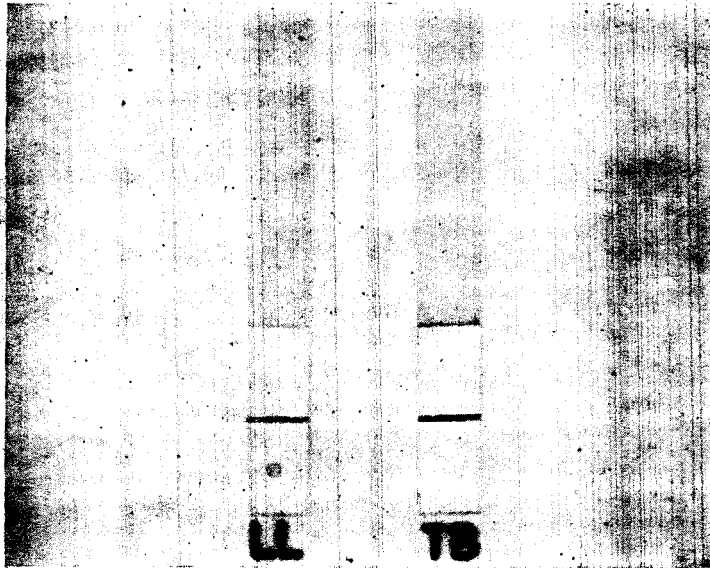


Fig. 1. PGL-I based dipstick dot EIA was highly specific for leprosy. A positive test with serum from lepromatous leprosy (LL) patient gave a blue dot against a clear white background of the lower nitrocellulose pad. The upper pad served as the internal reagent control. Sera from patients with active pulmonary tuberculosis (TB) were negative in the assay.

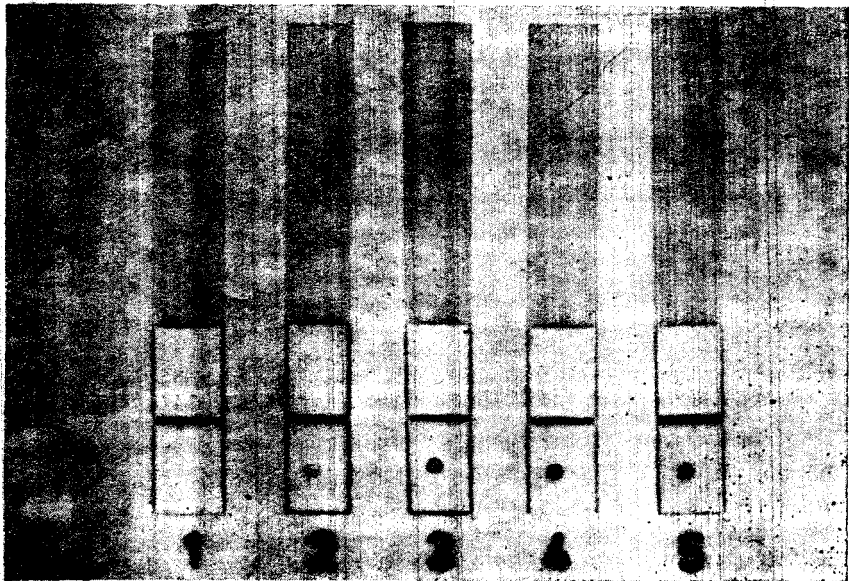


Fig. 2. The intensity of blue colour of the dot in dipstick assay varied from faint blue to deep blue (dipsticks 2-5), and it correlated with the gradation of anti-ND-BSA antibody (IgM) titres of these sera. Dipstick 1 shows a negative result.

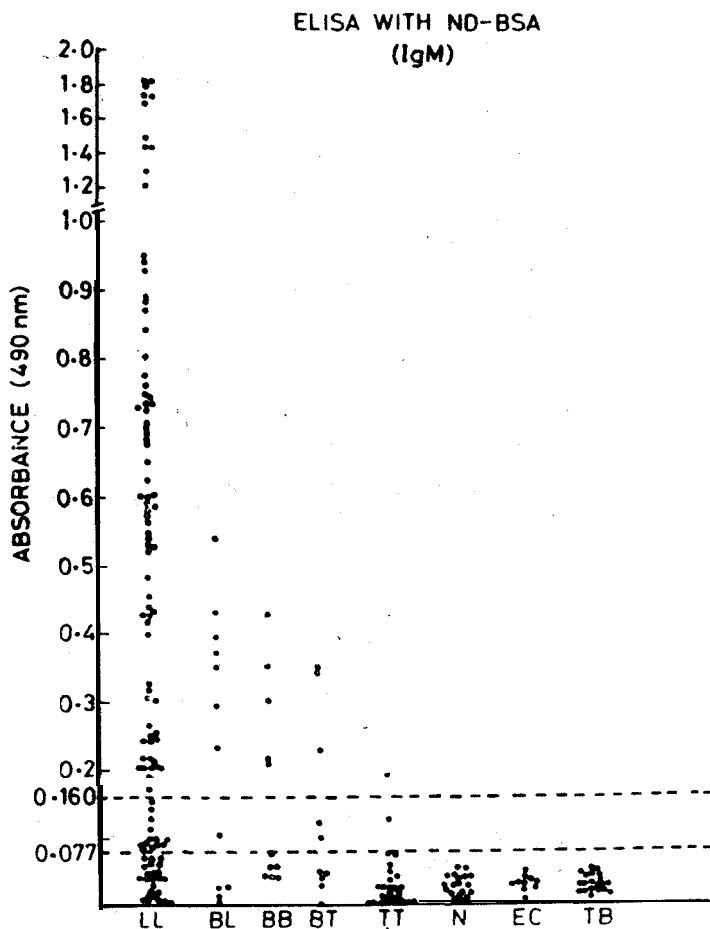


Fig. 3. A microtitre plate EIA based on synthetic-disaccharide (ND-BSA) was used for quantitation of IgM antibodies in the sera screened by dot EIA. Sera giving absorbance above 0.077 were considered positive in plate EIA. Most of the sera giving absorbance higher than 0.160 were positive in dot EIA.

or other mycobacteria do not interfere in the assay. The major limitation of the assay is its failure to put in evidence treated LL cases in whom the bacteriological index (BI) may have subsided substantially. A large number of tuberculoid leprosy patients are also not diagnosed by the assay (Figure 3). This test therefore can be primarily utilized for diagnosis of multibacillary (BL/LL) leprosy. Microtitre EIA plate technology has been adopted in many research laboratories. Its main advantage is in furnishing quantitative answers. EIA plates of requisite quality are not yet manufactured in the country and have to be imported. This factor alongwith the need for allied technology e.g. multichannel pipettes, plate washer and EIA reader being not available in most regional and peripheral laboratories, demand alternative simple approaches.

The present method uses indigenously made dipsticks. Nitrocellulose paper has good adsorption capacity for the antigen and antigen deposited is stable at room temperature for four months of observation period.

The dot assay gives visually readable results. With most of the sera positive by conventional EIA, where absorbance was above 0.160 by ND-BSA, were positive by dot EIA. Thus except for sera with borderline absorbance value in plate EIA, this assay is serviceable. The dipstick is easy to handle. However, further simplification of the procedure may be necessary for adoption of this technique by the field technician or paramedical worker. Preliminary results show that the two step incubation method as described here could be simplified to one step incubation, where conjugate and serum are added together. It will also be advantageous to employ simpler and stable substrate. These modifications are in progress.

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REFERENCES

1. ABE, M., IZUMI, S., SAITO, T. AND MATHUR, S.K. (1976). Early serodiagnosis of leprosy by indirect immunofluorescence. *Lepr. India*, **48** : 272-276.

2. AVRAMEAS, S. (1969). Coupling of enzymes to protein with glutaraldehyde. Use of conjugates for the detection of antigens and antibodies. *Immunochemistry*, **6** : 43.
3. BRETT, S.J., DRAPER, P., PAYNE, S.N., REES, R.J.W. (1983). Serological activity of a characteristic phenolic glycolipid from *Mycobacterium leprae* in sera from patients with leprosy and tuberculosis. *Clin. Exp. Immunol.*, **52** : 271-279.
4. CHO, S.N., YANAGIBARA, D.L., HUNTER, S.W., GELBER, R.H. AND BRENNAN, P.J. (1983). Serological specificity of phenolic glycolipid I from *Mycobacterium leprae* and use in serodiagnosis of leprosy. *Infect. Immun.*, **41** : 1077-1083.
5. FUJIWARA, T., HUNTER, S.W., CHO, S.N., ASPINALL, G.O. BRENNAN, P.J. (1984). Chemical synthesis and serology of the disaccharides and trisaccharides of phenolic glycolipid antigens from the leprosy bacillus and preparation of a disaccharide protein conjugate for serodiagnosis of leprosy. *Infect. Immune.*, **43** : 245.
6. HARBOE, M., CLOSS, O., BJUNE, G., KRONVALL, G. AND AXELSEN, N.H. (1978). *Mycobacterium leprae* specific antibodies detected by radioimmunoassay. *Scand. J. Immunol.*, **7** : 111-120.
7. RALHAN, R., BAND, A.H., ROY, A., HAJINI, G.H. SHARMA, A.K. AND TALWAR, G.P. (1985). An enzymeimmunoassay titrating IgM Antibody against phenolic glycolipid for diagnosis of lepromatous leprosy. *Indian J. Med. Res.*, **82** : 110-115.
8. SINHA, S., SENGUPTA, U., RAMU, G. AND IVANYI, J. (1983). A serological test for leprosy based on competitive inhibition of monoclonal antibody binding to the MY2a determinant of *Mycobacterium leprae*. *Trans. Royal Soc. Trop. Med. Hyg.*, **71** : 869-871.
9. YOUNG, D.B. BUCXANAN, T.M. (1983). A serological test for leprosy with a glycolipid specific for *Mycobacterium leprae*. *Science*, **221** : 1057-59.
10. YOUNG, D.B., FOHN, M.J., KHANOLKAR, S.R., BUCHANAN, T.M.(1985). A spot test for detection of antibodies to phenolic glycolipid I. *Lepr. Rev.*, **56** : 193-198.