HOW TO DO IT

A Low Budget DISC-ELISA for Hepatitis-B Surface Antigen (HBsAg)

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ELISA and Micro-ELISA
What is it ?

An enzyme linked immunosorbent assay (ELISA) described in 1971 for the detection of immunoglobulin-G (IgG) was similar to radioimmunoassay (RIA) but used an enzyme as a label instead of an isotope[1]. This technique received considerable attention in recent past including the detection of HBsAg[2,3] To reduce the cost, the test needs modification according to different requirements and conditions. Such a modification has already been done by us developing a micro-ELISA technique for the detection of HBsAg, where the commercially available reagents were used to combination with our preparations[4].

WHY ?

The ELISA kits available commercially in the market for testing of HBsAg including the techniques based on micro-ELISA are quite expensive. Every country has its own import policies, so in addition to its original cost, a large sum paid as tax and freight charges make test too expensive to use for routine purposes.

IN DEVELOPING COUNTRIES

In most of the developing countries where hepatitis B virus is a major cause of post-transfusion infection, routine screening of the blood donors for HBsAg is either not carried out at all or less sensitive techniques are being used. In fact high cost of the commercial kits are the major constraints

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and therefore low budget sensitive techniques are preferred.

Disc-ELISA

The present paper described the performance of Disc-ELISA on nitrocellulose membrane discs. The technique is essentially a modification of micro-ELISA where both the cost as well as time have been reduced significantly.

ELISA vs RIA
Stability
Shelf-life
Expense
Hazards

Some important features which make the ELISA more adaptable to certain laboratories than the RIA are enumerated as follows :

i) The enzyme is more stable than the isotope.

ii) The enzyme conjugate, if stored in proper conditions, is stable for two years where as lodine^{125} has a definite and limited half life of about 67 days.

iii) Expensive equipment like a gamma counter is not required.

iv) The test can be performed anywhere without radiation hazard.

COMMERCIAL CONJUGATE PREFERRED

The facilities for raising antibodies in experimental animals, their purification and conjugation with enzymes may not be available in a number of laboratories. Instead of using these sophisticated techniques, we have preferred to use freely available commercial anti-HBs-HRPO conjugate.

SOURCE OF COMMERCIAL KITS

Enzyme immunoassay kits for detection of HBsAg are commercially available from Abbott Laboratories, USA, Ortho-Diagnostics USA, Cordis Corporation USA, Organon Diagnostic Holland, the Behring Institute W. Germany and Sorin Biomedica Italy. Most of these establishments have outlet for their products in India. However, for the present test only Auszyme from Abbott Laboratories and Eliscaan from Sorin Biomedica obtained through Ranbaxy Laboratories have been used.

HOW
Test samples
Source
Storage

Blood samples received by the laboratories for liver function tests from the patient with liver diseases are collected and the sera stored at −75°C till the time the test is performed.

ENZYME CONJUGATE

1. **HRPO**
2. **Anti-HBs (Goat)**

Commercially available antibody to hepatitis B surface antigen (goat) conjugated to horse-radish peroxidase (HRPO) available from Abbott Laboratories is diluted to 1 : 10 with 50 per cent normal

human serum in phosphate buffer saline (PBS) just before use. Normal human serum is negative for HBsAg by the "Auszyme" and anti-HBs by the "Ausab" kits available for detection of HBsAg and anti-HBs respectively, from Abbott Laboratories. Normal human serum can be replaced with fetal calf serum, normal swine serum or normal horse serum without any appreciable change.

SUBSTRATE
Orthophenylene diamine (OPD)

Orthophenylene diamine (OPD), was used in a concentration of 0.4 mg per millilitre of substrate in 0.15M citrate buffer (pH 5.0). Hydrogen peroxide was added to the OPD solution to a final concentration of 0.006 per cent. The substrate is prepared fresh just before use and kept away from light.

PREPARATION OF DISC
Disc
Antibody
Concentration of antibody

The pre-coating of the antibody is carried out on 0.5 mm disc cut from sheets of nitro cellulose membranes (BA 83, 0.2 μm pore size obtained from Schleicher and Schull, Dassel, West Germany) Antibody to HBsAg, the antiserum, raised in horses to purified HBsAg, (Wellcome Labs. U.K.) with a protein content of 0.08 mg/ml, is tested at ten fold dilutions. The dilutions from 10^{-2} to 10^{-3} gives essentially the same result but the sensitivity in detecting HBsAg diminished at 10^{-4}. The antibody is diluted to a 1 : 500 for the purpose of coating of discs.

DILUENT
Carbonate buffer
pH 9.5 (0.1 M)
PBS pH 7.2
Normal saline

For pre-coating of the antibody to the disc, the three diluents generally used are 0.1 M carbonate buffer, pH 9.5; phosphate buffer saline (PBS), pH 7.2; and normal saline with 0.1 per cent sodium azide. We used carbonate buffer, pH 9.5, as the diluent for pre coating.

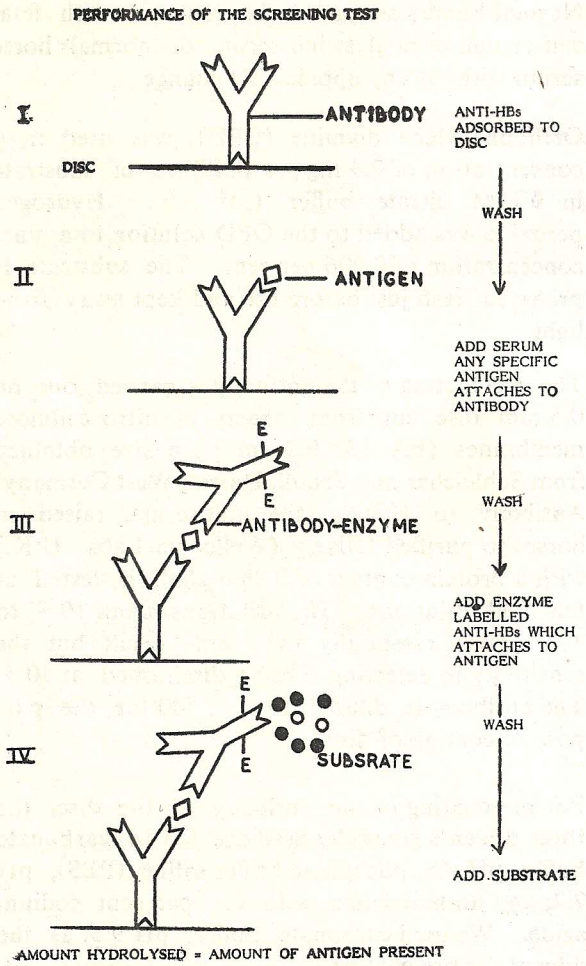
PRE-COATING
Activation
Quantity
Incubation

After initial incubation of the discs with 0.25 per cent glutaraldehyde, the discs are incubated with the antibody diluted to 1 : 500 in carbonate buffer at pH of 9.5, overnight at 4°C with continuous shaking on an end to end rotor. Excess volume of antibody is used so as to have optimal coating on both sides of the discs.

POST-COATING
BSA (1.5%)

After overnight incubation for pre-coating, the discs are washed thrice with PBS and incubated with 1.5 per cent bovine serum albumin (Sigma Chemical Co., USA) in PBS. They are incubated overnight at 4°C with continuous shaking on an end to end rotor to ensure complete blocking of

vacant sites on the disc. The discs are then washed with PBS as above and dried at room temperature. These discs can be stored at 4°C for over six months (unpublished obseration from our Lab).



During the test procedure the discs are washed with phosphate buffer saline, supplemented with 0.05 per cent Tween-20 (PBS-T)- Tween-20 is a detergent and helps in reducing the background activity for non-specific reactions.

As much as 50 μl of the test samples (positive and negative controls) are incubated with each disc coated with anti-HBs in an incubation tray at room temperature for 15 min. The discs are then washed once with PBS-T and 50 μl of the peroxidase conjugated anti-HBs are added to each well and incubated further at room temperature for 10 min. followed by five washings with PBS-T.

The discs are transfered to the micro-test plate (reusable) and 50 μl of freshly prepared substrate *i.e.* ortho-phenylene diamine in citrate buffer, pH 5.0 and hydrogen peroxide are added to each well.

After 3-5 min incubation at room temperature in a dark box, 100 μl of 2 M sulphuric acid is added to stop the reaction. The yellow colour of the product of the enzyme reaction is visualised by the naked eye against a white background of disc and/or by measuring the optical density of the end product at 492 nm in a micro-ELISA reader, after the disc has been removed from the well.

INTERPRETATION

Visual Observation :

Yellow color = Positive

In case O.D is measured :

$$\frac{\text{O.D. Test} - \text{O.D. Blank II}}{\text{O.D. Blank I} - \text{O.D. Blank II}}$$

O.D. of samples > O.D. mean negative control × 2.1

A sample is labelled positive if the yellow color is stronger than that of the negative control and/or the ratio of test to negative control, is more than 2.1 in case optical density (OD) is measured. The OD of blank I is the average optical density of 5-7 negative controls and OD of blank II is the optical density of 50 μl substrate and 100 μl of 2M sulphuric acid. A test sample with a ratio

between 1.5 and 2.1 is repeated and the test is considered negative if the ratio is still less than 2.1.

ADDITIONAL TECHNIQUES
Micro—ELISA
Auszyme

All the test samples were screened by disc-ELISA and micro-ELIAS[4]. Positive results were confirmed using the commercial kit "Auszyme" available from Abbott Laboratories, Chicago, Illinois, USA strictly following the manufacturer's instructions.

RESULTS COMPARISON
Disc ELISA 137/612 (22.4%)
Micro ELISA 137/612 (22.4%)
Results agreement = 100%

A total of 612 samples were tested for detection of HBsAg both by Disc-ELISA and micro-ELISA. Discrepancies in the results if any between two techniques were sorted out by using commercial kit Auszyme from Abbott Laboratory, USA. 22.4 per cent of the samples showed reactivity for HBsAg by both the techniques suggesting a complete agreement between the two. The difference in percentage positivity for detection of HBsAg by the two techniques was not significant. Both these tests are of equal sensitivity.

COMPARATIVE COST

DISC-ELISA	Micro-ELISA	Auszyme
Cost Rs. 2800/ 4000 tests	Rs. 5500/ 4000 tests	Rs. 2300/ 100 test
Cost/test Rs. 0.70	Rs. 1.40	Rs. 23.00
US cents 4.3	cents 8.8	Dollar 1.44

The cost per test for disc-ELISA comes out to be Rs. 0.70 (less than 5 US cents) which is just half the cost per test of Rs. 1.40 (less than 9 US cents, by the micro-ELISA and about thirtieth times less than the per test cost of Rs. 23.00 (about 1.5 US dollars) by the commercial macro-ELISA "Auszyme" kit. This cost is inclusive of all the test meterial required and received.

MERITS OF TEST
Cheap
Simple
Performance
Nitro cellulose membrane
PBS-T in washing solution

The Disc-ELISA is a cost effective test performed on nitro-cellulose membrane at room temperature. Merits of Nitro-cellulose membrane is that it has a better efficiency. Reaction can be viewed against a white background. The discriminatory power is greater. It binds proteins rapidly and quantitatively with high capacity. Initial activation of the nitrocellulose membrane with glutaraldelyde further increases the binding capacity. Addition of Tween-20 in a final concentration of 0.05 per cent in PBS during the test procedure reduces the background activity.

SPECIFICITY SENSITIVITY PRACTICABILITY

Post coating with 1.5 per cent BSA completely blocks the vacant sites which lowers the OD for the blanks thereby making the test more sensitive and diminishes the chances of false positives. The use of a heterologous antibody system *i.e.*, horse anti-HBs as a pre-coat and HRPO labelled goat anti-HBs as developing antibody has further reduced the non-specific cross reactions in the sandwich technique (Ab-Ag-AbE). The preliminary

results obtained with disc-ELISA and micro-ELISA on microtitre plates show that both the techniques have comparable sensitivity for detection of HBsAg. Earlier a report published by us has shown that micro-ELISA test has comparable sensitivity with that of commercially available kits "Auszyme".[4]

USEFULNESS
Simple and easy to perform
Sensitivity at low cost
Screening of blood donors
Follows WHO recommendations

The test is simple and easy to perform without the need of any sophisticated equipment. It has the same sensitivity at a thirtieth of the cost of commercial kits and half the cost of micro-ELISA tests. The technique is useful in screening any number of test samples within a limited budget.

In developing countries, like India, where the carrier rate for HBsAg is about 5 per cent, it becomes essential to screen all blood donors. The WHO recommendations[5] for screening of HBsAg in donor blood with sensitive technique could be followed up with the help of Disc-ELISA and micro-ELISA to a great extent.

LIMITING FACTOR
Use of commercial antibody conjugate

The main limiting factor in further reducing the cost of the tests is the use of the commercial antibody conjugate. Raising the antibody and preparing the antibody conjugate locally could further reduce the cost of the test. However, that remains a possibility only with commercial establishments, but it goes against their financial interest. Till the time, any voluntary organization or an Institute is prepared to share the responsibilities of preparing low cost reagents, it is felt that use of commercial conjugate will be much economical.

REFERENCES

1. Engvall E, Perlmann P. Enzyme linked immunosorbent assay (ELISA) quantitative assay of immunoglobulin G. *Immunochemistry* 1971; 8 : 871-4.
2. Wolters C, Kuigpers L, Kacaki J, Schuure A. Solid phase enzyme immunoassay for detection of hepatitis B surface antigen. *J Clin Pathol* 1976 29 : 873-9.
3. Wei R, Knight G J, Zimmerman D H, Bond H E. Solid-phase enzyme immunoassay for hepatitis B surface antigen. *Clin Chem* 1977; 23 : 813-5.
4. Gandhi BM, Tandon BN. A low cost micro-ELISA test for hepatitis-B surface antigen. *Trop Gastroenterol* 1984; 5 : 142-8.
5. World Health Organization : Viral Hepatitis (Report of a WHO meeting). *W H O Tech Rep Ser* 1975; 570.