HOW TO DO IT

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A low cost micro-ELISA test for hepatitis-B surface antigen

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ELISA
What is it ?

In 1971 Engvall and Perlmann[1] described an enzyme linked immunosorbant assay (ELISA) for the detection of Immunoglobulin-G (IgG). The test is similar to radioimmunoassay (RIA) but uses an enzyme as a label instead of an isotope. This technique has received considerable attention especially in the detection of HBs Ag. A solid phase 'sandwich' enzyme immunoassay for the detection of HBs Ag has already been described[2,3].

MICRO ELISA

In this article, we have discribed the performance of the micro-ELISA test on microtitre plates for the detection of HBsAg using the commercially available anti-HBs-HRPO conjugate thereby reducing to one-thirtieth the cost of each test.

WHY ?

The EIA kits available in the market are very expensive. Every country has its own import policies so, in addition to its original cost, a large sum is paid as tax and freight charges etc. The kits become too expensive to use for routine diagnosis and thus it has not been possible to use them for seroepidemiological surveys.

ELISA vs RIA
Stability
Expense
Hazards

Several studies[2,4,5] have shown that the enzyme linked immunosorbant assay (ELISA) and radioimmunoassay (RIA) are of equal sensitivity. There are some important features which make the ELISA technique more adaptable to certain laboratories than the RIA technique. The enzyme is more stable than the isotope. The enzyme conjugate, if stored in proper conditions, is stable for years where as Iodine 125 has a definite and limited half life. No expensive equipment like a gamma counter is required for the ELISA technique which can be performed in any laboratory anywhere without any radiation hazard.

COMMERCIAL CONJUGATE PREFERRED

The facilities for raising antibiodies 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 commercially available 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 and the Behring Institute W. Germany.

HOW
Test samples
Source
Storage

Blood samples from patients with acute viral hepatitis are collected and the serum samples 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 USA 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 Hepatitis B surface antigen and antibodies to Hepatitis B surface antigen respectively, from Abbott Laboratories USA.

SUBSTRATE
Orthophenylene diamine (OPD)

Orthophenylene diamine (OPD), which is superior to 5-amino salicylic acid because of its low rate of spontaneous colour change is used as a substrate. A concentration of 0.4 mg of OPD per millilitre of substrate is prepared in 0.15 M citrate buffer (pH 5.0). Hydrogen peroxide is 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 ANTIBODY MICROTEST PLATE
Plate
Antibody
Concentration of antibody

The pre-coating of the antibody is carried out in flat bottomed, 96 well microtest plates, obtained from Nunc Inter Med A/S, Denmark. Antibody to HBsAg received from Wellcome Labs. U. K. (the antiserum is raised in horses to purified HBsAg) 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 diminishes at 10^{-4}. The antibody is diluted to a 1 in 500 solution.

DILUENT
PBS pH 7.2
Carbonate buffer pH 9.5 (0.1 M)
Normal saline
with 0.1% sodium azide

For pre-coating of the antibody to the microtest plate, the nature of the diluent does not make any difference. The three diluents generally used are phosphate buffer saline (PBS) pH 7.2, 0.1 M carbonate buffer pH 9.5 and normal saline with 0.1% sodium azide. We use PBS as the diluent.

PRE-COATING
Quantity
Incubation

The antibody is diluted to 1 : 500 in PBS at a pH of 7.2 and 75 μl is added to the bottom of each well. The plates are incubated at 4°C in a mist chamber overnight for 18-20 hours.

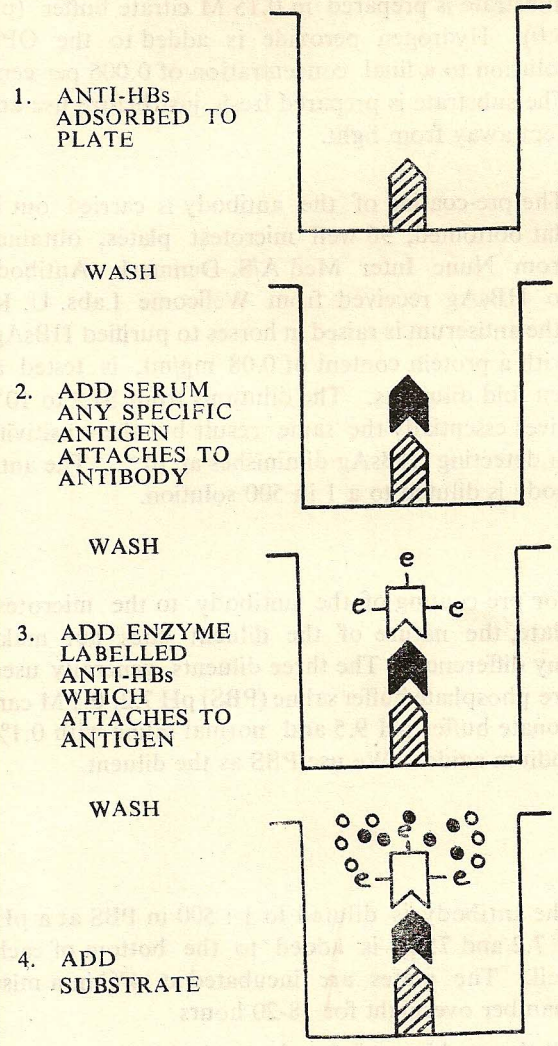
All the washings of the plates during the test procedure are carried out with phosphate buffer saline supplemented with 0.05 per cent Tween 20. Tween 20 which is a detergent helps reduce the background activity for non-specific reactions. A minimum of three washings is given for 5 minutes each.

POST-COATING
BSA (1%)

After overnight incubation for pre-coating, the plate is washed three times with PBS-T and into each well 200 μl of 1 per cent bovine serum albumin (Sigma Chemical Co USA in phosphate buffer saline is added and the plate incubated overnight at 4°C in a moist chamber.

FIGURE. 1.

PERFORMANCE OF THE SCREENING TEST



AMOUNT HYDROLYSED = AMOUNT OF ANTIGEN PRESENT

CALCULATIONS

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

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

25 μl of the test samples (positive and negative controls) are pipetted into the microtest plates coated with anti-HBs. The plates are incubated for 2 hours at room temperature, then washed three times in PBS-T and 50 μl of the peroxidase conjugated anti-HBs are added to each well. The plates are incubated at room temperature for 2 hours and washed five times with PBS-T.

100 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 30 minutes incubation at room temperature in a dark box, 75 μl of 2 M sulphuric acid is added to stop the reaction. The final volume of the reaction mixture is made to 250 μl by the addition of 75 μl of PBS. The yellow colour of the product of the enzyme reaction is read by the naked eye against a white background and/or by measuring the optical density at 492 nm in a spectrophotometer using a microcuvette or a microelisa reader.

A sample is labelled positive if the yellow colour is stronger than that of the negative control and if the optical density (O. D.), measured as the ratio of test to negative control, is more than 2.1. (The O. D. of blank I is the average optical density of 5-7 negative controls and O. D. of blank II is the optical density of 100 μl substrate, 75 μl of 2 M sulphuric acid and 75 μl of PBS). A test sample with a ratio between 1.5 and 2.1 is repeated and if the ratio is still less than 2.1, the test should be considered negative.

ADDITIONAL TECHNIQUES
Auszyme

To compare the results obtained by this modified technique against a standard technique, we screened the test samples against the commercial kit "Auszyme" available from Abbott Laboratories, Chicago, Illinois, USA strictly following the manufacturer's instructions.

RESULTS
COMPARISON
Auszyme 31/93 (33%)
ELISA 30/93 (32%)
on micro test plate
Difference—not significant

A total of 93 serum samples were tested for detection of HBsAg by Auszyme from Abbott Laboratories, USA and ELISA test using micro-titre plates. 32 per cent of the samples showed reactivity for HBsAg by the ELISA test on microtitre plates in comparison to 33 per cent by the commercial Auszyme test. Out of 93 samples tested, 33 were positive for HBsAg by Auszyme. All except one sample showed a positive test for HBs Ag by the micro-ELISA test. The difference in percentage of positivity for detection of HBsAg by the two techniques was not significant. Both these tests are therefore of equal sensitivity.

COMPARATIVE COST

	Auszyme	ELISA on micro test plate
Cost	Rs. 4500/ 100 tests	Rs. 5500/ 4000 tests
Cost per test	Rs. 45.00	Rs. 1.40

The cost per test is Rs. 1.40 or (US $ 0.14) by the micro-ELISA method which is very much cheaper than the cost per test of Rs. 45.00 (US $ 4.5) by the commercial Auszyme kit. This cost is inclusive of all the test material required and received.

MERITS OF TEST
Simple
Irradiation of plates
for better coating
Storage in humidified chambers
PBS-T in washing solution

The micro-ELISA test is a simple test performed on micro titre plates. The plates from M/s Nunc, Inter Med A/S Denmark are received sterilised by irradiation. However, further activation of these plates under UV light for 3 to 4 hours just before coating with anti-HBs results in a better binding of antibody. The incubation of the plate in a humidified chamber increases the storage life of the plates for the test. Addition of Tween 20 in a final concentration of 0.05 per cent in PBS reduces the background activity.

SPECIFICITY SENSITIVITY
PRACTICABILITY

Post coating with 1 per cent BSA further lowers the O. D. 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 horse radish peroxidase (HRPO) labelled goat anti-HBs has further reduced the non-specific false reactions resulting from cross reactions in the sandwich technique (Ab-Ag-AbE). The preliminary results obtained with micro-ELISA test on microtitre plates show that this technique for detection of HBsAg has a sensitivity that compares very favourably with that of commercial available kits "Auszyme".

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. The technique is most useful in screening a large number of test samples within a limited budget.

In developing countries, like India, where the carrier rate for HBsAg is above 5 per cent, it become essential to screen all blood donors. Due to a lack of funds, all the blood which is being transfused is either not being screened or being screened by insensitive techniques. The WHO recommendations[6] that the tests for screening of HBsAg in donor blood should be as sensitive as possible, are not being followed. The micro-ELISA test reported here may help solve the problem.

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 will further reduce the cost of the test.

We recommend the micro-ELISA technique as an easy and highly sensitive low cost test for the detection of HBsAg in serum.

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