TROPICAL GASTROENTEROLOGY-VOL. 6, NO. 3, 1985

HOW TO DO IT

B. M. GANDHI
M. IRSHAD
B. N. TANDON

A low cost micro-ELISA test for detection of antibodies to hepatitis-B surface antigen

Rajgarhia Liver Research Unit,
Department of Gastroenterology & Human Nutrition, All-India Institute of Medical Sciences, New Delhi, India

ELISA

Detection of immunoglobulin G (IgG) by an enzyme linked immunosorbant assay (ELISA) dates back to 1971. Since then the technique has been used to detect a number of antibodies. Detection of antibodies to HBsAg requires purified antigen for binding on the solid phase and as a carrier for enzymes. The facilities for purification of HBsAg are not available in most laboratories and it also poses a threat of infection. An effort has been made by us to solve both these problems. Purified HBsAg protein from HBsAg vaccine has been used as a binder protein on the solid phase and protein-A has been used to replace purified HBsAg as the carrier protein.

MICRO ELISA

In this article, we have described the performance of the micro-ELISA test on microtitre plates for the specific detection of IgG antibodies to Hepatitis B surface antigen using the commercially available vaccine for HBsAg as the source of purified antigen and enzyme linked protein-A as the conjugate-thereby reducing the cost of each test with an increase in specificity.

WHY
Micro-ELISA ?

The EIA kits available in the market are expensive. Even though the import policies with respect to diagnostic kits for HBsAg have been relaxed and these kits are exempt from payment of customs duty, the test is beyond the budget of many laboratories in developing countries. The kits to test anti-HBs are also expensive and cannot be used for routine hospital screening or seroepidemiological surveys.

WHY
Test for anti-HBs ?

Antibody to Hepatitis B surface antigen is a long persisting antibody associated with convalescence and recovery from hepatitis B infection. The presence or absence of this antibody determines the status of individuals with type B viral hepatitis. A positive anti-HBs test is useful for assessing the clinical recovery of the patient. In addition, the screening of anti-HBs is useful for assessing the immunity against HBsAg for immunization. The study of anti-HBs can help identification of epidemiological factors associated with transmission of HBsAg and estimate the endemicity of hepatitis B in the population.

COMMERCIAL ANTIGEN PREFERRED

The setting up of the technique for the detection of antibodies to HBsAg is subject to the availability of purified antigen as a pre-coat on the solid phase. The facilities for purification of HBsAg are not available in a number of laboratories. Even if the facilities are available, workers do not wish to risk iufection with HBsAg. Commercially available H.B. Vax from Merck Sharp & Dohme which contains inactivated HBsAg, has been used by us as the source of antigen for the pre—coat. HB Vax is relatively non-infectious and is now easily available at most places. The vaccine is prepared without regard to subtype, so it can be used to detect IgG antibodies to HBsAg including all known subtypes-with the present ELISA system.

Source of Commercial Kits

Enzyme immunoassay kits for detection of antibodies to HBsAg are commercially available from Abbott Laboratories, USA, Ortho-Diagnostic. Holland and the Behring Institute, West Germany.

HOW
test samples our stored

Blood samples from different groups and subjects are collected and the serum samples stored at-75°C till the time the test is performed.

ENZYME CONJUGATE
HRPO
Protein-A

Commercially available Protein-A from *Staphylococcus aureus* (Sigma, P-6650) is labelled with horse radish peroxidase (HRPO) (Sigma, P-3375) by the method of Engvall (1980) using a two step technique with glutaraldehyde. The enzyme conjugate is diluted 1:2000 in phosphate buffer saline pH 7.4 containing 10% foetal calf sera. The dilution of conjugate is obtained by checker board titration.

CHOICE OF ENZYME CARRIER
Protein-A

The proteins used as carriers for the enzyme have been either specific antibodies or anti-immunoglobulins, which should be monospecific or monoclonals. Protein-A obtained from *Staphylococcus aureus* with its extra-ordinary affinity for Fc regions of IgG subclasses 1.2 and 4 of most of the tested species, is used as the enzyme carrier. It is a single polypeptide chain of molecular weight 42,000 and can be easily labelled with HRPO. The advantages of use of protein A for conjugation over immunoglobulins is that this molecule is more stable to heat and denaturing agents due to its smaller size. It can be easily coupled to the enzyme and the binding to IgG is complete and quick within seconds.

SUBSTRATE
Orthophenylene diamine (OPD)

Orthophenylene diamine was used as a substrate for horse radish peroxidase (HRPO). A concentration of 0.4 mg of OPD per millilitre of substrate is prepared in 0.15 M citrate buffer, pH 5.0 (10.3 ml of 0.2M Na_2HPO_4 and 9.7 ml of 0.1 M citric acid). 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 ANTIGEN
MICROTEST PLATE
Plate
Antigen
Concentration of antigen

The pre-coating of the antigen is carried out in flat bottomed 96 well microtest plates obtained from Nunc Inter Med A/S. Denmark. HBsAg vaccine H B Vax from Merck Sharp and Dohme, USA., with a protein concentration of 20 μg per ml is tested in dilutions of 2 μg, 1 μg. 0.5 μg and 0.1 μg per ml. The concentration of antigen at a dilution of 1 μg/ml gave the maximum optical density. The HBsAg antigen is diluted to a 1 μg/ml solution.

DILUENT
0.1M Carbonate buffer pH 9.5
PBS pH 7-2
Normal saline with azide

For precoating of the antigen to the microtest plate, the nature of the diluent does not make any difference. The three diluents used are 0.1 M carbonate buffer pH 9.5, phosphate buffer saline (PBS) pH 7.2 and normal saline with 0.1% sodium azide. We use carbonate buffer as the diluent which contains 27 ml of 0.1 M Na_2Co_3 and 73 ml of 0.1M $NaHCo_3$.

PRE-COATING
Quantity, Incubation

The HBsAg from the vaccine is diluted to 1 μg/ml in 0.1M carbonate buffer, pH 9.5 and 50 μl is added to the bottom of each well in the microtest plate. The plate is incubated at room temperature 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 (PBS-T). 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.

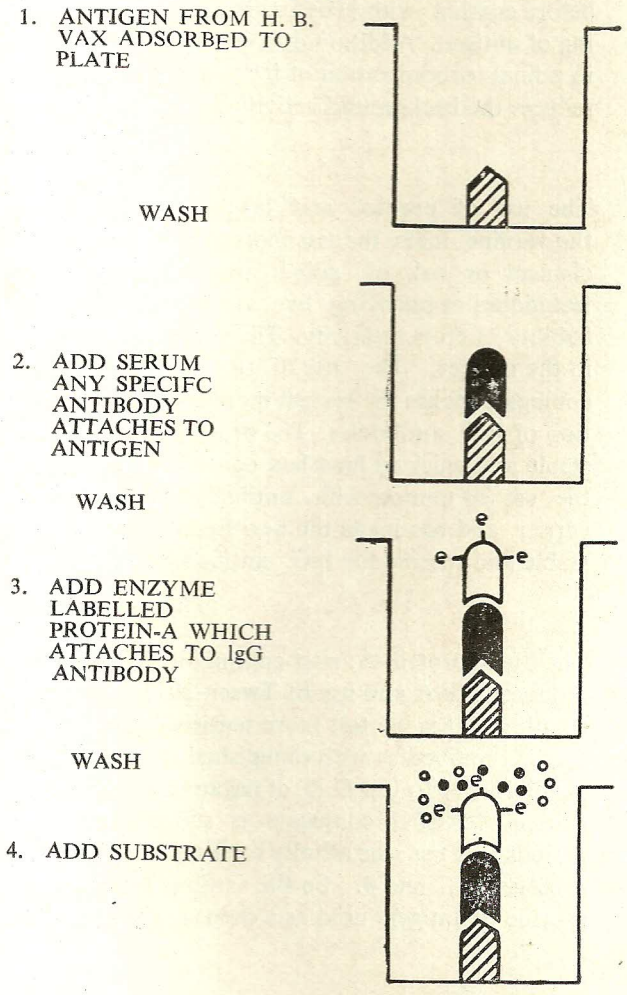
POST-COATING
Gelatin (0.5%)
BSA (1.0%)

Two different blocking agents, Gelatin (0.5%) and bovine serum albumin (1%) used did not make any difference. Gelatin being cheaper is used. After overnight incubation for pre-coating, the plate is washed three times with PBS-T. Into each well, 200 μl of 0.5% gelatin (Sigma Chemical Co. USA) in phosphate buffer saline is added and the plate incubated for 90 min at 37°C.

PERFORMANCE OF THE SCREENING TEST

50 μl of the diluted serum or plasma in PBS, and negative controls are pipetted into the microtest plates coated with HBsAg. The plates are incubated for 2 hours at 37°C, then washed three times in PBS-T and 50 μl of the protein-A-HRPO conjugate in a dilution of 1:2000 is added to each well. The plate is incubated at 37°C for 1 hour and washed five times with PBS-T.

FIGURE - 1.
PERFORMANCE OF THE SCREENING TEST



AMOUNT HYDROLYSED = AMOUNT OF IgG ANTIBODY PRESENT

100 µl of freshly prepared substrate i. e. orthophenylene diamine in citrate buffer pH 5.0 and hydrogen peroxide are added to each well. 100 µl of 4 M sulphuric acid is added to stop the reaction after 5 to 30 min., depending on the colour of the mixture. The yellow colour of the product of the enzyme reaction is read by measuring the optical density at 492 nm in a micro-ELISA reader or by the naked eye against a white background.

CALCULATIONS

OD negative control ± 2 SD

A sample is labelled positive if the optical density (0 D.) measured is more than the optical density of negative controls plus 2 S.D. A minimum of 8 samples which are negative for anti-HBs are run as negative controls with each batch of 96 tests in a microtest plate. For visual observation, a sample is labelled positive if the yellow colour is stronger than that of the negative controls.

ADDITIONAL TECHNIQUES

AUSAB

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

MERITS OF TEST
Simple Quick
Irradiation of plate for better Coating
Washings with PBS-T

The micro-ELISA test is a simple and quick test performed on micro titre plates. Activation of these plates under UV light for 3-4 hours just before coating with HBsAg results in better binding of antigen. Addition of Tween-20, a detergent, in a final concentration of 0.05 per cent in PBS reduces the background activity

MERITS OF REAGENTS USED
HBsAg from vaccine
Protein A-HRPO Conjugate

The use of purified and inactivated HBsAg in the vaccine make the test more safe to use with no chances or risk of going through cumbersome techniques of purifying live virus and a low probability of cross reactivity. The vaccine is available in the market. The use of the protein-A HRPO conjugate makes the system more specific for detection of IgG antibodies. The protein-A being more stable and quick to bind has conveniently replaced the use of monospecific antibody as an enzyme carrier and has made the new ELISA system more stable and specific for IgG antibodies.

SPECIFICITY SENSITIVITY
PRACTICABILITY

The use of protein-A, post coating of the plate with 1 per cent BSA and use of Tween-20 in the wash solution makes the test more sensitive and specific for IgG antibodies with diminished chances of false positivity due to low O.D. of negative blank samples. Protein-A-HRPO conjugate is stable for longer periods and has the affinity for Fc regions of IgG subclasses 1,2 and 4. So the entire IgG contents specific to antigen used as a precoat are picked up.

COMPARATIVE COST

	AUSAB-EIA	*MICRO ELISA*
COST	*Rs 3000 100 test*	*Rs 900 1200 tests*
Cost per test	*Rs 30.00*	*Rs 0.75*

The cost per test in Rs. 0.75 (US $ 0.06) by the micro-ELISA method which is much cheaper than the cost per test of Rs. 30.00 (US $ 2.4) by the commercial AUSAB-EIA kit. This cost is inclusive of all the test material required

RESULTS
Comparison

AUSAB-EIA	*18/52 (35%)*
Micro-ELISA	*18/52 (35%)*

Out of 52 samples tested from different disease groups, 18 samples were found to be positive for antibodies to HBsAg by both the techniques i. e. commercially available AUSAB-EIA from Abbott Laboratories USA and the present micro-test plates. Both these techniques are found to be equally sensitive.

USEFULNESS
Simple and easy to perform
Sensitivity at low cost
No risk of infection
Seroepidemiological studies
Monitoring for immunization

The test is simple and easy to perform without the need of any sophisticated equipment. Sensitivity is maintained at a fortieth of the cost of commercial kits. The technique is most suitable for screening a large number of test samples for seroepidemiological surveys, to study the endemicity of the hepatitis B infection in the population. Besides revealing the association of antibody with convalescence from hepatitis B virus infection, the test is useful to monitor the immunization against Hepatitis B virus infection in individuals.

LIMITING FACTOR
Availability of Vaccine
ELISA reader

The main limiting factor in the system is the availability of vaccine. The present system is more suited to developing countries where a large sum of money cannot be spent on the purchase of commercial reagents. The vaccine may not at present be freely available in most of the countries. The other factor involved is the ELISA reader which is beyond the scope of an average laboratory. In visual observations, one has to compromise with very weak positive reactions.

The micro-ELISA system described in the text is an easy and highly sensitive low cost test and is recommended for the detection of antibodies to Hepatitis B surface antigen (HBsAg).