# Hepatitis B surface antigen in donated blood — screening and confirmation by enzyme-linked immunosorbent assay

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## Summary

Principles of and test procedures for enzyme-linked immunosorbent assay (ELISA) screening and confirmatory tests for hepatitis B surface antigen (HBsAg) in donated blood are described. The assay is of 'third-generation' sensitivity and is practical and economical for large-scale screening of blood donors. Results from 119 000 tests show ELISA to be superior to the passive haemagglutination inhibition (PHAI) test for HBsAg. The ELISA described here is considerably cheaper than any comparable commercially available isotopic kit for HBsAg screening but is slightly less sensitive.


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The discovery of what is today known as hepatitis B surface antigen (HBsAg),[1] a surface component of hepatitis B virus,[2,3] was a major breakthrough in the history of viral hepatitis. Pre-transfusion screening of donated blood, introduced in the late 1960s, reduced the incidence of post-transfusion hepatitis.[4] This disease has been one of the major hazards connected to blood transfusion.

Considerable advances have been made in the last 10 years in the development of sensitive, rapid and practical methods for the detection of HBsAg. Today 'third-generation' assays are freely available and are widely used for the screening of donated blood. Of the 'third-generation' methods, immunoradiometric assay (IRMA), which employs a 'sandwich' principle, is generally accepted as the most sensitive method.[5,6] Unfortunately the cost of commercially available kits and equipment makes this assay prohibitive for most blood transfusion services in South Africa.

The development in 1971 of enzyme-linked immunosorbent assay[7,8] (ELISA) and its use for the detection of HBsAg[9] offer a cheaper alternative to the isotopic assays. The popularity of ELISA stems mostly from the possibility that this kind of assay can be developed 'in house'. Furthermore, less expensive equipment is required and the enzyme-labelled antibody has a longer shelf-life than its isotope-labelled equivalent.[10]

We describe ELISA screening and confirmatory tests for HBsAg in donated blood which have been in use since July 1980, present results obtained from preliminary studies and compare ELISA with two other 'third-generation' methods.

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## Material and methods

### Isolation of HBsAg

HBsAg was prepared from donated blood heavily contaminated with hepatitis B virus. Typically, HBsAg in 1 litre of serum was precipitated by addition of PEG 6000 (11% w/v) and the precipitate recovered by centrifugation at 1 000 *g* for 10 minutes. The precipitate was redissolved in 500 ml 0,05M tris-0, 1M NaCl buffer, pH 8,0 (TS), and centrifuged at 176 000 *g* for 90 minutes in a Spinco rotor 60Ti.[11] Pellets were resuspended and pooled in a total of 100 ml TS and centrifuged again at 176 000 *g* for 90 minutes, after which pellets were collected and resuspended in 12 ml NaBr at $\rho = 1{,}30$. Gradient centrifugation on an NaBr gradient between density 1,28 and 1,15 took place at 82 000 *g* for 16 hours in a Spinco SW27 rotor.[12] Fractions of 0,5 ml were collected after bottom-puncture of the centrifuge tubes and the density of each fraction was determined. Those fractions having a density between 1,20 and 1,24 were pooled and dialysed against TS, and the hepatitis B virus was concentrated by centrifugation at 176 000 *g* as before. The resulting pellet was resuspended in TS, carefully layered onto a 7,5 - 20% (w/v) sucrose gradient and centrifuged in a Spinco SW27 rotor for 16 hours at 82 000 *g*. The pellet resulting from this was resuspended in 65% sucrose in TS and overlayered by a 20 - 60% (w/v) sucrose gradient. After centrifugation at 82 000 *g* for 16 hours (SW27 rotor) the position of the HBsAg band was determined by measuring the absorbency at 280 nm. Purified HBsAg was pooled, dialysed against TS and stored at −20°C. An $\epsilon^{0,1\%}_{280nm} = 3{,}73$[12] was used to determine the concentration of HBsAg.

### Production of sheep anti-HBsAg

Sheep were immunized by intramuscular injection at multiple sites with 50 μg HBsAg in Freund's complete adjuvant for 4 weeks at weekly intervals. After 6 weeks test blood samples were obtained and sera were examined for the presence of anti-HBs. Sera produced in this manner were also tested by immunoelectrophoresis for the presence of antibodies against human serum proteins. Such antibodies were routinely removed from immune serum by passage through a column (5 x 90 cm) of human serum insolubilized onto Sepharose 6B.[13] The immunoglobulin fraction of antiserum was recovered by precipitating three times with 15% (w/v) sodium sulphate. The final precipitate was dissolved in a minimum volume of distilled water and extensively dialysed against distilled water. Euglobulins which formed were removed by centrifugation and the clear supernatant was freeze-dried.

### Conjugate preparation

Highly purified horseradish peroxidase (HRPO type VI; Sigma) was used. Antibody HRPO conjugate was prepared according to the method of Wilson and Nakane.[14] Normally 8 mg sheep IgG was conjugated to 4 mg horseradish peroxidase. After conjugation the mixture was chromatographed on a column (2,6 x 83 cm)

of Sephacryl S-300 in TS. The absorbancy was monitored spectrophotometrically at 280 nm and 403 nm. Selected fractions containing conjugate were pooled and mixed with an equal volume of glycerol containing 0,02% (w/v) $NaN_3$. Conjugate was stored at 4°C (it should not be deep-frozen).

## Antibody coating of microtitre plates

The concentration of sheep antibody used in coating plates was established by developing ELISA colour in a plate coated with increasing amounts of antibody. The concentration of antibody at which a plateau in ELISA colour was reached was used for coating subsequent plates. Throughout this study plates were coated at a concentration of 80 μg/ml. Dynatech 129B plates were coated with 100 μl sheep antibody using a semi-automatic dispenser (Titertech Autodrop; Flow Laboratories). Antibody was dissolved in 0,05M glycine-0,1M NaCl buffer, pH 2,5, and after 5 - 10 minutes was brought to a pH of 7 - 7,5 by the addition of solid tris before the plates were coated.[15] Plates were allowed to stand for 2 hours at room temperature before washing by means of a specially constructed washing 'shower',[16] first with TS containing 0,05% Tween 20 (TST) and then with distilled water. Coated plates were dried under vacuum and packed and sealed into airtight plastic-metal-foil laminate packets containing a small bag of silica gel desiccant.

## ELISA methodology

The ELISA for the detection of HBsAg in donated blood consists of a screening assay which is followed by a confirmation assay on positive samples.

### *Screening assay*

Dispensing of conjugate, chromogenic substrate and acid-stop was done with a Titertech Autodrop using separate syringe heads for each reagent, as follows:

1. Pipette accurately 50 μl of each of two positive controls (positive +++, positive +) into each of three adjacent wells and 50 μl of a negative control serum into 5 additional wells.
2. Dispense 50 μl of individual samples to be tested into available wells.
3. Cover the plate with a lid and place in a sealed humidified container. Incubate at 45°C for 90 minutes.
4. Wash plate once with TST using the washing 'shower'.
5. Dispense 50 μl of diluted anti-HBs-HRPO conjugate to each individual well. Conjugate is diluted in TS containing 10% (v/v) neutral sheep serum and 5% (v/v) negative human serum.
6. Cover the plate with a lid, place in a humidified container and incubate at 45°C for 60 minutes.
7. Wash plate as before with TST. Dispense 50 μl chromogenic substrate* to each well and allow colour to develop in the dark for 30 - 40 minutes at room temperature (20 - 22°C).
8. Stop the enzymic reaction by adding into each well 100 μl of 1,5N HCl.
9. Measure the intensity of developed colour (492 nm) in a Dynatech MR 580 Microelisa Autoreader. Serum samples giving an absorbancy three times greater than that of the mean of the negative controls are presumed to be positive for HBsAg. A Prime I1000 computer was linked to the photometer and was used to directly calculate positive/negative (P/N) values obtained in the assay. Results were printed out on a Texas Instruments 820 printer.

This assay normally takes 3 hours.

### *Confirmation assay*

1. Dispense 50 μl of presumptive HBsAg-positive sample into each of two adjacent wells of an anti-HBs-coated plate.
2. Cover the plate with a lid, place in a humidified container and incubate overnight (18 hours) at room temperature.
3. Wash plate once with TST using the washing 'shower'.
4. Add 50 μl of human anti-HBs antiserum to the first well and 50 μl of 4% (w/v) human serum albumin in TS to the second well.
5. Incubate in a humidified container at 45°C for 90 minutes.
6. Follow procedure as for screening assay starting at point 4.

A sample is confirmed positive if the absorbancy of the well treated with human anti-HBs antiserum is reduced by 50% or more relative to the absorbancy in the companion well treated with HSA.

### *Immunoradiometric assay for HBsAg*

Commercial kits using $^{125}I$ as label were purchased from Travenol and samples were tested for the presence of HBsAg according to the manufacturers' instructions.

### *Passive haemagglutination inhibition (PHAI for detecting HBsAg)*

This assay was developed at the Natal Blood Transfusion Service[17] and has been in routine use for screening donated blood since 1977.

# Results

## Determination of the cut-off point of the ELISA

Serum samples from 2 946 blood donors known to be free from HBsAg were collected and tested by ELISA in groups of 200 -300 together with 15 - 20 negative controls (2% (v/v) neutral sheep serum in TS) per group. Fig. 1 shows the distribution of the P/N ratio of the serum samples. Because of the skewed nature of this distribution it was apparent that the high P/N ratio of certain negative samples would overlap with that of weakly positive samples (Fig. 2). In order to determine the lowest detectable P/N ratio of a positive sample, which is signifi-

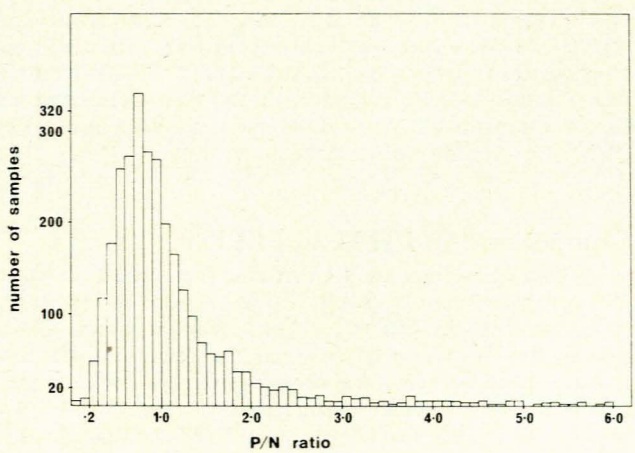


**Fig. 1. Frequency histogram of P/N ratios in an HBsAg-negative population.**

*Chromogenic substrate was prepared from anhydrous borax ($Na_2B_4O_7$) 93 mg, succinic acid 93 mg; urea hydrogen peroxide 10 mg, and distilled water 25 ml. To this was added 30 mg *o*-phenylenediamine dihydrochloride (Fluka) which had been washed in methylene chloride containing 5% (v/v) MeOH until a clear filtrate was obtained. This ratio of borax and succinic acid should yield a pH of 5,0. Fine white particles in the solution originate in the urea hydrogen peroxide and need not be removed. Because of the catalytic breakdown of $H_2O_2$ by metals it is important that contact with metals be completely avoided during all steps of the assay, and buffer salts used in the substrate should be of sufficient purity to avoid development of high background colour. The purity of the hydrochloric acid used is particularly important because acid of low quality will cause spurious colour development. Merck Suprapur HCl was found to be excellent.

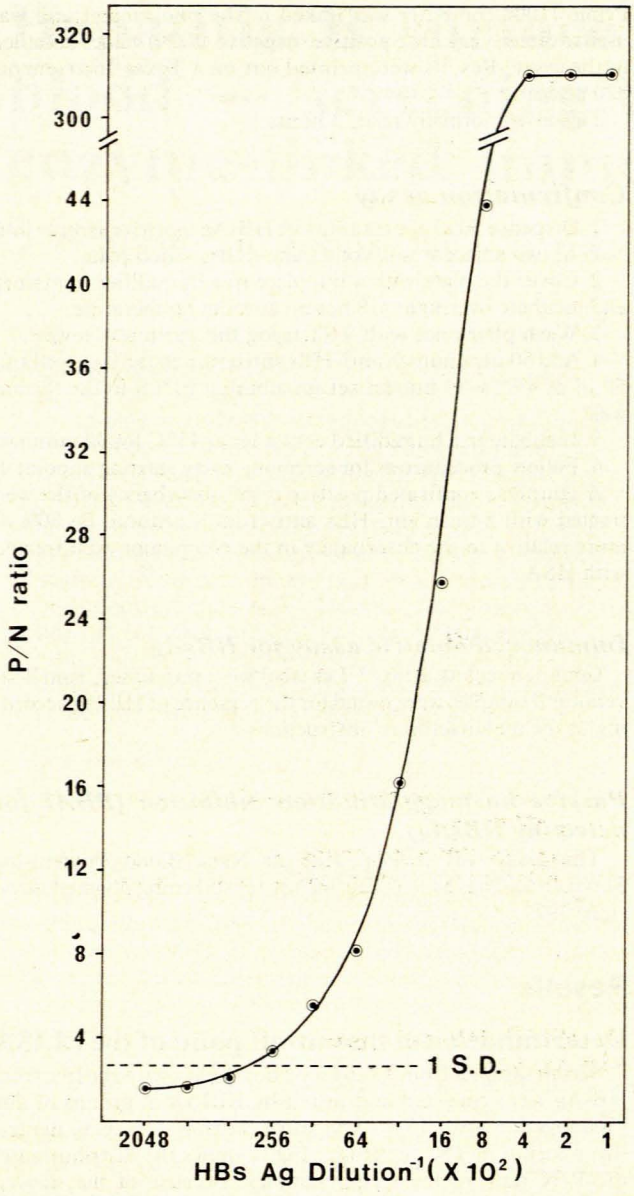


**Fig. 2. Standard curve showing P/N ratio v. HBsAg dilution.**

cantly higher than the mean negative control, an HBsAg-positive serum sample was serially diluted and tested by ELISA. The dose-response curve obtained is shown in Fig. 2. Analysis of replicate samples at each dilution on this curve show that the absorbancies differed by more than 1 SD from the negative mean from dilutions of 1/25 600 or less. In this study values above 1 SD or approximately three times the negative control mean (P/N = 3) were therefore regarded as positive.

## Comparison of PHAI and ELISA

In order to evaluate the ELISA described here 9 701 blood donor samples were screened for the presence of HBsAg by PHAI and ELISA (Table I). This preliminary trial clearly showed the ELISA to be as specific as the PHAI but also underlined the fact that it was necessary to retest fewer samples. This preliminary comparison indicated that the ELISA was a more objective and reliable assay than PHAI and that a further trial was warranted.

Table II shows results from a much larger series of blood samples tested by ELISA. These included new and old blood

**TABLE I. RANDOM BLOOD DONOR SAMPLES TESTED BY PHAI AND ELISA**

| | PHAI | ELISA |
|---|---|---|
| No. tested | 9 701 | 9 701 |
| No. negative | 8 880 | 9 531 |
| No. retested | 821 (8,46%) | 170 (1,75%) |
| No. positive | 52 | 51 |
| No. repeatedly false positive | 1* | 0 |

*Confirmed negative by IRMA.

**TABLE II. LARGE-SCALE SCREENING BY ELISA FOR HBsAg IN DONATED BLOOD**

| | | % of total |
|---|---|---|
| No. tested | 118 532 | 100 |
| No. negative | 111 931 | 94,4 |
| No. retested by confirmatory assay | 6 601 | 5,6 |

donors, males and females of the four racial groups constituting the donor population in Natal. In this series 118 532 samples were tested by 45 individual technicians over a period of 18 months. Of these samples 94,4% were negative by the ELISA screening test and 5,6% were presumed positive and had to be retested by the ELISA confirmatory test (Table III). This test showed that 5 121 (77,6%) of the samples were in fact negative and that 910 were positive. The remaining 570 were considered 'inconclusive' because of technical problems or 'borderline' P/N values. Upon retesting these 570 samples with IRMA, 16 were found to be positive.

**TABLE III. SAMPLES WITH P/N ⩾ 3,0 RETESTED BY ELISA CONFIMATORY ASSAY (BLOCKING ASSAY)**

| | | % | % of total |
|---|---|---|---|
| No. tested | 6 601 | 100 | 5,6 |
| No. negative | 5 121 | 77,6 | 4,3 |
| No. positive | 910 | 13,8 | 0,8 |
| No. inconclusive | 570 | 8,6 | 0,5 |

The sensitivities of ELISA and IRMA were compared by testing serial doubling dilutions of two strongly positive HBsAg sera of the two main subtypes 'ad' and 'ay'. The lowest dilutions which still gave a positive result are shown in Table IV. Although IRMA detected both subtypes with equal facility, ELISA displayed different sensitivities towards the subtypes and was also less sensitive than IRMA.

## Discussion

The ideal assay for detecting HBsAg in blood should be: (*a*) sensitive; (*b*) rapid; (*c*) objective, i.e.leaving no room for technician error; (*d*) inexpensive; and (*e*) stable, i.e. having a long shelf-life.

No single assay satisfies the above requirements, and for that reason several different types of assays using different detection principles have been developed. Immunoassays using isotopic labels are known to be very sensitive, but they have the drawback of being relatively expensive and special laboratory equipment

**TABLE IV. SENSITIVITY OF ELISA AND IRMA (TRAVENOL) IN DETECTING HBsAg OF SUBTYPES ad AND ay**

| HBsAg | ELISA titre$^{-1}$ | IRMA titre$^{-1}$ |
|---|---|---|
| **ad** | **8 000 (3,5)** | **16 000 (5,4)** |
| **ay** | **4 000 (4,1)** | **16 000 (3,2)** |

P/N values in parentheses.

and disposal facilities are required. Assays dependent on haemagglutination or inhibition of haemagglutination are generally not as sensitive as IRMA but have the attraction that they are usually more rapid. Unfortunately results from these assays are more difficult to interpret, resulting in a lack of objectivity, especially by inexperienced staff.

We have elected to develop an ELISA for detecting HBsAg in donated blood because this method is thought to be as sensitive as IRMA and conforms to most of the requirements set out above. In addition it readily lends itself to automation and the reagents used can easily be produced in most laboratories.

It is evident from Table I that PHAI and ELISA have the same sensitivity for the detection of HBsAg. However, ELISA has the advantage over PHAI that almost all the samples could be tested, fewer samples had to be retested, and the ELISA confirmation test uses a different blocking antibody from that used in the conjugate. It is also important to note that it normally takes 3 - 4 weeks to train technicians to interpret PHAI results accurately, whereas the same people can be trained for the ELISA within 4 - 5 days. This is mostly because results are scored photometrically and therefore in an objective manner.

Over a period of 18 months the ELISA described here screened out 5,6% of samples of donated blood as being potentially HBsAg-positive (Table II). This percentage is considerably higher than that in the smaller series summarized in Table I and probably reflects the fact that 45 technicians were involved in the running of this trial. This discrepancy can therefore be attributed mostly to inexperienced staff. Nevertheless it is of interest to note that false-positive rates of 5 - 8% have been reported for two commercial IRMA kits.[18] In our experience this ELISA is almost as sensitive as a commercial IRMA (Travenol). Similar sensitivity is possible with ELISA but requires longer incubation times or amplification of the system through addition of another antibody layer in the 'immune sandwich'. Both these possibilities are impracticable in a blood bank where the screening for HBsAg of donated blood must be completed as soon as possible. Yet another possibility exists, and this lies in the production of monoclonal antibodies of high avidity. We have recently developed a mouse hybridoma producing monoclonal $IgG_1$ antibodies, and preliminary results with this material are very encouraging.

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