# Hepatitis B Virus Antigen Detection by Reverse Passive Hemagglutination (37408)

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Numerous immunologic methodologies have been applied in detecting hepatitis B virus associated antigens (HB Ag) in human blood (1-7). Although the reverse passive hemagglutination (RPHA) reaction with its high sensitivity and simplicity in operational procedure appears to be an attractive candidate as diagnostic aid, it has not received much attention. We report here a practical procedure for detecting HB Ag by the use of erythrocytes coated with antibody to HB Ag. The RPHA was found to be one of the most sensitive among the five methodologies explored.

*Hepatitis B Virus Associated Antigen (HB Ag).* Human plasma samples judged positive for HB Ag[1] were obtained from commercial blood donors. HB Ag was purified in general according to the procedure reported by Gerin *et al.* (8). Alternate isopycnic banding in cesium chloride and sucrose density gradient sedimentation were repeated to give the antigen preparations with less than 5% contamination of human serum proteins as estimated by quantitative agar gel diffusion technique. The concentration of purified HB Ag was estimated by the biuret reaction using bovine serum albumin as standard. A mixture of ad and ay subtypes of HB Ag were present in the preparations (7). The purified HB Ag was supplied by Dr. L. R. Overby and Dr. C. M. Ling.

*Antisera to HB Ag.* Antisera were produced in guinea pigs by the multiple route/multiple site procedure which comprised injection of 0.5 ml (50–100 μg) of the purified HB Ag in an equal volume of Freund's complete adjuvant using intravenous, intraperitoneal, intramuscular, and intradermal routes. The animals received booster injections in the same manner about 10 weeks later, except that the iv injection was omitted. They were bled four weeks later. A sera pool was made which had double agar diffusion (DAD) titer of 1/256 to 1/512 against the ad subtype.

*Fractionation of Anti-HB Ag Sera.* Antisera were fractionated by Sephadex G200 chromatography. Antibody was found in both the 7 S and 19 S fractions as determined by the passive hemagglutination reaction using HB-Ag-coated human type O erythrocytes. The 7 S fraction had 100–1,000 times the titer of 19 S fraction.

*Double Agar Diffusion (DAD), Counter-immunoelectrophoresis (CEP), and Complement Fixation (CF) Tests.* These tests were performed essentially according to the procedures described in WHO Bulletin (9).

*Hemagglutination Inhibition (HAI) Test.* The passive HA test was performed according to the procedure described previously (10). In brief, human type O erythrocytes were stabilized by treatment with pyruvic aldehyde followed by formaldehyde. The stabilized erythrocytes were designated as FPHE. Packed FPHE (0.1 ml) was resuspended in 10 ml of 0.1 *M* acetate buffer, pH 4, containing 200 μg of purified HB Ag and stirred for 5 min at room temperature. HB-Ag-coated FPHE (HB-FPHE) was resuspended in 0.11 *M* phosphate buffer, pH 7.2. The HA reaction was performed on plastic trays with 96 "V" bottom wells (Linbro Chemical Co., New Haven, CT, Cat. IS-MVC-96, disposable). The diluent for the antiserum comprised of the phosphate buffer containing 1

[1] Detected positive by DAD, CEP, or CF using AUS-tect™ antiserum for HB Ag, Abbott Laboratories, North Chicago, Illinois.

TABLE I. RPHA Titers of Selected Hepatitis-Suspect Sera.

| RPHA titer range | ≤16 | 64–16,000 | 32,000–128,000 | >128,000 |
|---|---|---|---|---|
| No. of sera in range | 33 | 22 | 20 | 11 |

mg/ml gelatin (Difco Co., Detroit, MI) and 0.5 mg/ml of rabbit serum albumin. The HAI procedure was essentially according to Vyas and Shulman (3), except that antiserum was used at 4 HA units (four times the concentration of the passive HA titer of the serum).

*Reverse Passive Hemagglutination Test.* The procedure for coating FPHE with 7 S fraction of guinea pig anti-HB Ag serum was similar to that for coating FPHE with HB Ag as described in the HAI section. Packed FPHE (0.1 ml) was resuspended in 10 ml of 0.1 *M* acetate buffer, pH 4, containing 300 μg of the 7 S fraction and stirred for 75 min at room temperature. Coated FPHE was resuspended in phosphate buffer, pH 7.2. Serial twofold dilutions of purified HB Ag or patient's sera were made in 0.11 *M* phosphate buffer, pH 7.2, containing 0.1% gelatin and 1% normal human serum. One drop (about 25 μl) of diluted HB Ag or serum was added to plastic microtiter plate. One drop of 0.25% antibody-coated FPHE was added to each well. The titer was read as the reciprocal of the highest dilution which varied significantly from the control (diluent only).

Initially, 14 sera were selected from hepatitis suspects which were negative in the DAD test. Most of the sera were also negative (titer of 16 or less) in the RPHA test, but surprisingly four gave titers of 16,000. In a more extensive survey, 86 serum samples from hepatitis suspects were examined by the RPHA. As shown in Table I, the titers ranged from less than 16 to greater than 128,000.

In these studies, uncoated FPHE served as controls. Titers of serum samples were 16 or less. A further study on control titers was made using 37 HB Ag positive and 20 negative sera against uncoated FPHE and FPHE coated with the 7 S fraction from normal guinea pig serum. Samples of positive serum were selected from those which were positive as judged from DAD and RPHA data. As data in Table II indicate, most of sera whether HB Ag positive or negative gave titers of less than 16. There was no serum with a titer of 64 or above.

When five different methods were compared in the detection of purified HB Ag, the DAD was the least sensitive and the RPHA the most sensitive (Table III). The comparative sensitivity of the various methods, excluding the RPHA, is similar to that found by other investigators (1, 3, 7, 11).

Juji and Yokochi reported on an RPHA procedure using formaldehyde and tannic-acid-treated erythrocytes coated with antibody purified from human serum (12). They dissociated HB Ag-antibody complex by 3 *M* NaI and separated antibody and antigen by Sephadex chromatography. They reported the highest positive titer as 2048 and relatively high nonspecific titers of up to 1024 where erythrocytes coated with normal human γG were used as control. Sera from patients

TABLE II. HA Titers of HB Ag Positive and Negative Human Sera Against Uncoated Human FPHE and FPHE Coated with Normal Guinea Pig Gamma Globulin (GPGG).

| | Sera, HB Ag Positive or Negative | | | | | |
|---|---|---|---|---|---|---|
| | Positive | Negative | Positive | Negative | Positive | Negative |
| | HA titer, reciprocal | | | | | |
| | <16 | | 16–32 | | ≥64 | |
| | No. of sera | | | | | |
| FPHE | 8 | 25 | 2 | 2 | 0 | 0 |
| GPGG-FPHE | 17 | 4 | 3 | 6 | 0 | 0 |

TABLE III. Comparison of Six Different Methods for the Detection of Hepatitis B Virus Associated Antigen.

| Methods | Detection limit of HB Ag[a] (ng/test vol) | (ng/ml) | Test volume (μl) |
|---|---|---|---|
| Double agar diffusion | 2000 | 200,000 | 10 |
| Counterimmunoelectrophoresis | 10 | 2000 | 5 |
| Complement fixation | 40 | 1600 | 25 |
| Passive hemagglutination inhibition | 20 | 800 | 25 |
| Reverse passive hemagglutination | 0.04 | 1.6 | 25 |

[a] Purified HB Ag was diluted by serial twofold dilutions in 1% normal human serum diluted in phosphate buffer.

may well contain rheumatoid factor and may agglutinate erythrocytes coated with human γG. The use of guinea pig antibody may have an advantage in this regard. The use of guinea pigs as a source of antibody has an additional advantage in that hyperimmune sera can be obtained. When hyperimmune serum is used, erythrocytes can be coated with a crude fraction (7 S) instead of isolated antibody. The high sensitivity obtained in the present method is attributed mainly to the use of erythrocytes treated by the double aldehyde procedure (10).

Although a high sensitivity was attained by RPHA, further study is necessary to ascertain whether equal sensitivity can be obtained with antibody to various HB Ag subtypes (13).

*Summary.* A reverse passive hemagglutination (RPHA) procedure was developed for the detection of hepatitis B virus associated antigen (HB Ag) in human serum. The comparative sensitivity of six different methods was double agar diffusion<counter immunoelectrophoresis<complement fixation <passive hemagglutination inhibition< RPHA.

1. Blumberg, B. S., Sutnick, A. I., London, W. T., and Millman, I., "Australia Antigen and Hepatitis." Chemical Rubber Co., Cleveland (1972).

2. Mayumi, M., Okochi, K., and Nishioka, K., Vox Sang. **20**, 178 (1971).

3. Vyas, G. N., and Shulman, N. R., Science **170**, 332 (1972).

4. Fritz, R. B., and Rivers, S. L., J. Immunol. **108**, 108 (1972).

5. Vyas, G. N., Williams, E. W., Klaus, G. G. B., and Bond, H. E., J. Immunol. **108**, 1114 (1972).

6. Stevens, R. W., Ziegler, F. D., and Kelly, J. H., J. Immunol. **108**, 1123 (1972).

7. Ling, C. M., and Overby, L. R., J. Immunol. **109**, 834 (1972).

8. Gerin, J. L., Holland, P. V., and Purcell, R. H., J. Virol. **7**, 567 (1971).

9. Bull. WHO **42**, 957 (1970).

10. Hirata, A. A., and Brandriss, M. W., J. Immunol. **100**, 641 (1968).

11. Schmidt, N. J., Gee, P. S., and Lennette, E. H., Appl. Microbiol. **22**, 165 (1971).

12. Juji, T., and Yokochi, T., Jap. J. Exp. Med. **39**, 615 (1969).

13. Holland, P. V., Purcell, R. H., Smith, H., and Alter, H. J., J. Immunol. **109**, 420 (1972)

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