Journal of Hepatology, 1986; 3: 356–362
Elsevier

HEP 00229

Rotative Agitation RIA

A High Sensitivity Technique for Hepatitis B Surface Antigen (HBsAg) Determinations

J.A. Pineda, F. García de Pesquera, A. Sánchez Quijano, M. Carmona, R. Castro, M. Leal and E. Lissen

Departments of Internal Medicine, Nuclear Medicine and Hematology, Virgen del Rocio Hospital, Seville (Spain)

(Received 20 March, 1986)
(Accepted 8 July, 1986)

Summary

With the objective of finding a simpler and more sensitive method than conventional radioimmunoassay testing to detect HBsAg, we have modified the sample volume and first incubation procedure of the Ausria IIA method. There was an up to 9-fold increase in sensitivity without loss of specificity, in different assays, when the first incubation was carried out with a serum sample of 1 ml that was rotatively agitated for 22 h at room temperature.

Additionally, 71 samples from which conventional testing had isolated hepatitis B core antibodies were tested with the modified rotative agitation technique and 9 of these specimens (12.67%) were positive for HBsAg. Routine employment of the rotative agitation radioimmunoassay technique to screen HBsAg by blood banks, to detect potential infectious donations that escape conventional testing, could be an effective as well as low-cost procedure to prevent post-transfusion hepatitis B.

Introduction

Solid-phase radioimmunoassay (RIA) testing is a highly sensitive and specific method to determine HBsAg. Its routine use by blood banks, in conjunction with the exclusive acceptance of voluntary blood donations, has managed to significantly reduce post-transfusion hepatitis B (PTHB) [1]. Nevertheless, PTHB still represents almost 10% of all the post-transfusion cases in the United States [2,3], and an even higher percentage in our country [4–6]. The theory that blood with a low level of HBsAg, that was not detectable by conventional RIA analyses, was responsible for some of these cases was tossed around

Correspondence address: Dr. Juan A. Pineda, Departamento de Medicina Interna, Hospital Virgen del Rocio, Avda Manuel Siurot, S/N 41013 Sevilla, Spain.

for years [7], before being subsequently proven [8,9]. Likewise, blood with hepatitis B core antibodies (anti-HBc) when hepatitis B surface antibodies (anti-HBs) are absent carries a particularly high transfusional risk [7,10,11]. When blood samples with isolated anti-HBc by commercial RIA techniques have been retested for HBsAg by more sensitive methods, some specimens have been shown to contain low levels of this marker, which confirms the potential infectivity of these samples [8,9,12–14]. Therefore, it would be highly recommendable for blood banks to use methods with greater sensitivity than the RIA assays that are available at present. Nath et al. have already shown that simple modifications to conventional RIA incubation procedures can increase their sensitivity by as much as 2.8-fold [12].

We have developed a method, based on a few simple changes in the manner that the first incubation of a commercial RIA test for HBsAg is carried out, which has increased its sensitivity by an extraordinary degree. Moreover, we have tested isolated anti-HBc serum samples by this new method in order to prove its efficiency.

Material and Methods

(1) Adapting the modified technique

Changes in the sensitivity of the Ausria IIA method (Abbott Labs, North Chicago, U.S.A.) were assessed, after the following modifications were made to the manufacturer's specifications: (a) the length of the first incubation was increased to 4, 8, 12, 18, 20 and 22 h; (b) the incubation was carried out at 4 °C, 22°–24 °C (room temperature) and at 45 °C, with and without lengthening the time of the first incubation; (c) the first incubation was carried out at rest and both using an end-over-end rotation and a rotative agitation of the mixture methods; (d) the volume of the sample was increased up to 1.4 ml.

The rotation and the rotative agitation of the incubation mixture were performed by a laboratory rotator (ATOM-100) and a rotative agitator (SI-100, Pharmacia Diagnostics) respectively at a speed of 28 revolutions per min. The rotator shifted the mixture in the tube end-over-end while the rotative agitator moved along a horizontal plane and followed the outline of a square which thereby rotated the solid-phase at the bottom of the capped glass vials. After the first incubation was finished, the beads were transferred to the wells in the trays that are provided in the Ausria II kits. The second incubation was then carried out at 45 °C. The norms recommended by the manufacturer were followed for the rest of the procedure.

The introduction of these modifications resulted in a very sensitive technique with which the following experiments were carried out.

(2) Sensitivity and specificity evaluation of the modified technique

The sensitivity of the modified technique was assessed and compared to the conventional method by testing successive dilutions of a standard serum obtained through the courtesy of Abbott Laboratories, the HBsAg/ay content of the dilutions was estimated to range from 7.5 ng/ml to 1 ng/ml. To verify the increase in sensitivity, we analyzed a pool of 30 sera that had been found to be HBsAg-positive by conventional RIA and dilutions of this mixture were tested by both methods. An additional 9 samples, that were also HBsAg-positive according to the standard technique, were tested separately. In order to investigate the specificity of the modified method, these 9 samples were blocked with cold anti-HBs and then retested. All of the sera used were voluntarily donated to our blood bank. A calibration panel, supplied by Abbott Laboratories, was used as the standard to estimate the amount of HBsAg in the sera and in the dilution panels. The amount of antigen estimated, ranged from 2.65 to 0.04 ng/ml for HBsAg/ad and from 1.66 to 0.09 ng/ml for HBsAg/ay.

(3) HBsAg determinations by the modified technique using sera with isolated anti-HBc

Seventy-one serum samples that were negative for HBsAg and anti-HBs but positive for anti-HBc by commercial RIA were taken from our blood bank and tested for IgM anti-HBc and HBsAg by the modified method. These samples had remained frozen, at −25 °C, from the moment they were obtained until

they were retested by the modified method. When they were unfrozen, their prior condition of being only reactive for anti-HBc by conventional RIA, was confirmed in all of them.

(4) Marker determinations using commercial RIA

Following the manufacturer's instructions, HBsAg, anti-HBs, anti-HBc and IgM anti-HBc were tested by solid-phase RIA using commercial reagents facilitated by Abbott Laboratories (Ausria II, Ausab, Corab and Corab M, respectively). In all positive cases, a second determination, using the same method, was carried out in order to confirm the results.

Results

(1) Achievements of the high-sensitivity technique

Changes in incubation time and temperature, while the other guidelines of the standard method remained unaltered, did not promote any apparent improvement in sensitivity. When the first incubation was carried out with only end-over-end rotation there was also no appreciable improvement in sensitivity. However, when rotative agitation was employed, the sensitivity of the method was enhanced 2–3-fold. When the sample volume was also increased, the sensitivity became more and more marked until a maximum of 1 ml was reached, but beyond this point, there was no further improvement. An increase in the amount of serum used did not determine any enhancement of sensitivity when the standard method was employed (Fig. 1). When rotative agitation and 1 ml of serum was employed, changes in the incubation times and the temperatures did not promote any significant changes in the sensitivity of the method.

Hence, we selected rotative agitation which we shall refer to as RA-RIA and sample volumes of 1 ml for the first incubations. In the experiments carried out with this technique, the first incubation was left for 22 h at room temperature.

(2) RA-RIA sensitivity and specificity

The actual sensitivity of the RA-RIA method was determined by using a serum that had a HBsAg ay concentration of 15 ng/ml (standard from Abbotts

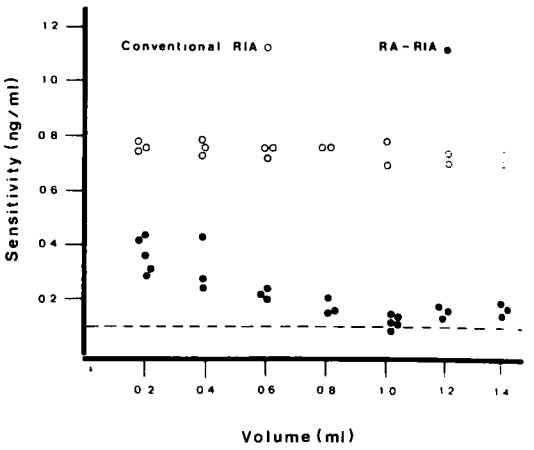

Fig. 1. Effect of sample volume on sensitivity. Each data point represents an individual experiment and each sample was tested in triplicate.

TABLE 1

SENSITIVITY COMPARISONS OF STANDARD AND ROTATIVE AGITATION TECHNIQUES

The serum sample was from a commercial sensitivity panel and each dilution was tested in triplicate.

Sample dilution	HBsAg/ay concentration (ng/ml)	Conventional RIA (+/−)	RA-RIA (+ −)
1/2	7.50	12.4	70.4
1/4	3.75	6.9	46.8
1/8	1.87	4.4	25.8
1/10	1.50	4.6	23.0
1/12	1.25	4.6	20.4
1/16	0.94	2.8	15.7
1/20	0.75	2.6	13.0
1/22	0.68	2.0	12.2
1/24	0.63	1.2	11.2
1/30	0.50	–	10.8
1/40	0.38	–	8.0
1/50	0.30	–	7.2
1/60	0.25	–	5.4
1/80	0.19	–	4.8
1/100	0.15	–	4.4
1/120	0.12	–	2.8
1/130	0.12	–	2.4
1/140	0.11	–	2.3
1/150	0.10	–	1.3

TABLE 2

SENSITIVITY COMPARISONS OF CONVENTIONAL AND RA-RIA FOR HBsAg/ad

Each sample was tested in triplicate.

HBsAg/ad (ng/ml)	Conventional RIA (+/−)	RA-RIA (+/−)
2.65	7.3	37.9
2.13	6.8	35.2
1.71	5.8	24.1
1.07	4.1	20.6
0.74	3.0	12.3
0.36	2.4	10.5
0.16	1.5	4.9
0.04	1.6	2.3

Labs.) that was successively diluted from 1/2 to 1/150. The results are shown in Table 1. The RA-RIA tests were positive even for concentrations as low as 0.11 ng/ml where as the conventional method had a cutoff point of 0.75 ng/ml. A calibration panel was used as the standard to estimate the antigen levels of the samples in the dilution panels. The conventional technique detected a mean minimal level of 0.36 ng/ml for HBsAg/ad and 0.74 ng/ml for HBsAg/ay while the RA-RIA method was positive up to 0.04 ng/ml for the ad subtype and 0.09 ng/ml for the ay subtype (Tables 2 and 3). This represents a 9-fold sensitivity improvement for HBsAg/ad and 8.2-fold increase for HBsAg/ay.

TABLE 3

SENSITIVITY COMPARISONS OF CONVENTIONAL AND RA-RIA FOR HBsAg/ay

Each sample was tested in triplicate.

HBsAg/ay (ng/ml)	Conventional RIA (+/−)	RA-RIA (+/−)
1.66	5.5	23.0
1.34	4.4	20.2
1.05	4.2	13.0
0.74	3.2	11.2
0.54	2.0	10.8
0.36	2.0	18.0
0.23	1.8	5.4
0.18	1.1	4.8
0.09	1.0	2.4
0.00	1.0	1.0

TABLE 4

ANALYSIS RESULTS OF DIFFERENT DILUTIONS OF HBsAg POSITIVE SERUM MIXTURES USING CONVENTIONAL AND RA-RIA

Each dilution was tested in triplicate.

Dilutions	Conventional RIA (+/−)	RA-RIA (+/−)
1/100	28.6	102.7
1/200	20.4	69.7
1/400	13.3	66.9
1/600	8.1	46.7
1/800	5.5	25.9
1/3200	3.3	12.7
1/6400	1.9	9.9
1/12800	1.7	10.9
1/25600	1.2	3.8
1/51200	1.1	2.0

The analysis results of the dilutions of the 30 HBsAg positive pool are shown in Table 4. When 9 HBsAg sera were tested separately, a higher titre of this marker was found upon employing RA-RIA in all these samples (Table 5). These same sera were later blocked with cold anti-HBs globulins and reanalyzed by RA-RIA and they all turned out to be negative for HBsAg.

(3) HBsAg determinations using RA-RIA of sera with anti-HBc isolated by conventional RIA

The 71 samples containing anti-HBc isolated by conventional RIA were still in their original serologi-

TABLE 5

RESULTS OF THE TITRATION OF 9 DIFFERENT SERA USING CONVENTIONAL RIA AND RA-RIA

Serum number	Conventional RIA titre	RA-RIA titre
1	1:1600	1:12800
2	1:6400	1:25600
3	1:25600	1:102400
4	1:12800	1:51200
5	1:12800	1:102400
6	1:1600	1:12800
7	1:3200	1:25600
8	1:1600	1:6400
9	1:1800	1:6400

TABLE 6

S/N RATIOS BY CONVENTIONAL RIA AND RA-RIA FOR HBsAg IN 9 ANTI-HBc-POSITIVE SERUM SAMPLES

Each sample was tested in triplicate by both methods.

No.	Conventional RIA	RA-RIA
1	1.5	6.1
2	1.4	6.6
3	1.9	8.4
4	2.0	3.2
5	1.6	12.8
6	1.0	5.1
7	1.1	5.4
8	1.8	20.3
9	1.7	6.8

cal condition once they were unfrozen and were IgM anti-HBc-negative. When they were tested by RA-RIA, 9 of them (12.67%) were found to be HBsAg-positive. The sample-to-negative control (S/N) averages obtained by conventional RIA and RA-RIA test are itemized in Table 6.

Discussion

The results have shown that RA-RIA remarkably improves the sensitivity of the conventional method. In addition, it is a relatively simple technique and has a similar cost. At the same time, as other authors have already proven [8,9,12,14], our study has made it clear that at least some blood donations with isolated anti-HBc contain low levels of HBsAg that are not detectable by conventional RIA screening. As a consequence, some donors that are potential transmitters of hepatitis B (HBV) have been accepted by our blood banks. This would explain, at least, a part of the PTHB cases that continue to appear in spite of the exclusive acceptance of donations that are HBsAg-negative by conventional RIA testing.

Rotative agitation of the incubation mixture brings about a greater contact between its elements, which can increase the sensitivity of the technique [15]. For this same reason, it should follow that end-over-end rotation should also enhance its sensitivity. However, we found that foam was produced, due to the speed of the agitator used in our laboratory, and this made it difficult for the serum and the anti-HBs bound to the solid-phase to make contact. However, the SI 100 rotative agitator, due to the different way in which it revolved, did not produce foam even though it was run at the same speed as the rotator. Thus, the problem that rotation presented was solved.

As serum volume increases, so does the number of HBsAg particles and this can cause additional interactions between these particles and the solid-phase antibodies which, therefore, should improve the sensitivity of the method. Theoretically, this should also imply that a greater number of non-specific reactions, owing to the presence of a large number of particles that cross-react with the HBsAg molecules, should decrease the specificity of the method. However, when assaying was repeated after blocking the HBsAg particles with anti-HBs, this did not come about.

The exclusion of donor blood containing isolated anti-HBc, detected by conventional RIA, as a measure to prevent PTHB has been highly controversial [7–9,16,17]. The adoption of this policy, in an area such as ours, would make it necessary to turn away a sizeable number of donors as well as bring about additional laboratory expenses which are not easy to absorb by blood banks [18,19]. Therefore, every effort has been made to identify blood donations which contain isolated HBc that pose a true transfusional risk. In theory, the presence of isolated anti-HBc in blood can be the expression of at least three very different situations as far as HBV infections are concerned: a recent 'window phase' infection; a carrier with persistent HBV that has a small amount of HBsAg circulating in his blood; or a resolved HBV infection in which anti-HBs levels have become undetectable by RIA [7,20].

In order to differentiate the first two subgroups, which are potentially infectious, from the third that theoretically does not carry any potential risk, it has been proposed that IgM anti-HBc as well anti-HBc titres should be determined and then the blood donations that have shown IgM anti-HBc-positive results

or high titres of anti-HBc should be excluded. However, both courses pose serious problems, not only for the expense involved, but also for the lack of sensitivity of the first method and the lack of sensitivity and specificity of the second to identify blood donations included in the second subgroup [18,20–22].

Therefore, the solution to the problem of identifying donor blood with isolated anti-HBc and residual infectious ability will in all likelihood only come about when blood banks employ techniques with a sensitivity higher than that of commercial RIA for HBsAg to detect HBV infections.

For this reason, several tests have been developed that have a greater sensitivity than the standard RIA for HBsAg. Among these, the method based on selective enzymatic digestion of a sample with pepsin and its subsequent concentration with polyethyleneglycol should be pointed out [23]. Although this technique is highly sensitive, it is too complex for routine blood bank use. Methods based on rotation of the incubation mixture, such as those reported by Nath et al. [12] and Park [24], have proven to enhance the sensitivity of conventional RIA testing for HBsAg. However, RA-RIA does have a sensitivity that is 3–4-fold greater than the first of these and does not require complicated laboratory equipment or large sample volumes as in the second. Wands et al. have developed a multiple site monoclonal 'simultaneous sandwich' RIA which uses IgM anti-HBs (5D3) [13,14,25–28]. This method has made it possible to detect HBsAg determinants in up to 26% of blood samples containing isolated anti-HBc by conventional RIA [13,14]. Unfortunately, this method has yet to be placed on the market so it is only at the reach of a very few centers. On other hand, the specificity of monoclonal RIA testing could be questionable, since recent studies have shown that this technique not only detects HBsAg, but antigenic determinants of a virus that is immunologically different from HBV [29]. The RA-RIA technique has been shown initially as a specific method. Additionally it can be at the reach of any center that has the facilities to carry out conventional RIA.

We believe studies should be carried out to compare not only the sensitivity and specificity but the cost-benefit and cost-efficiency relationships between the monoclonal RIA and RA-RIA methods, in order to determine the feasibility of their employment by blood banks.

Since RA-RIA testing has been effective in detecting very small levels of HBsAg in carriers' blood, its routine use in blood bank screenings could prove to be a practical as well as cost-efficient measure to prevent PTHB.

References

1 Aach RD, Kahn RA. Post-transfusion hepatitis: current perspectives. Ann Intern Med 1980; 92: 539–546.
2 Aach RD, Lander JJ, Shermann LA, et al. Transfusion transmitted viruses: interim analysis of hepatitis among transfused and non transfused patients. In: GN Vyas, SN Cohen, R Schmid (Eds.), Viral Hepatitis. Franklin Institute Press, Philadelphia, 1978: 393–396.
3 Alter HJ, Holland PV, Purcell RH, et al. Post-transfusion hepatitis after exclusion of commercial and hepatitis B positive donors. Ann Intern Med 1972; 77: 691–699.
4 Hernandez JM, Piqueras J, Carrera A, Triginer J. Post-transfusion hepatitis in Spain. A prospective study. Vox Sang 1983; 44: 231–237.
5 Bárcena R, Suarez E, Gil Grande L, et al. Estudio prospectivo de la hepatitis postransfusional ni A- ni B. Incidencia y factores de riesgo. Gastroenterol Hepatol 1983; 6: 1–4.
6 Sánchez Quijano A, Lissen E, Castro R, et al. Hepatitis postransfusional en Sevilla. Incidencia, distribución etiológica y profilaxis con immunoglobulina inespecífica de reciente elaboración. Gastroenterol Hepatol 1984; 7: 321–325.
7 Hoofnagle JH, Seef LB, Bales ZB, Zimmerman HJ and the Veterans Administration Hepatitis Cooperative Study Group, Type B hepatitis after transfusion with blood containing antibody to hepatitis B core antigen. N Engl Med 1978; 288: 1379–1383.
8 Hopkins R, Kane E, Robertson AE, Haase G. Hepatitis B virus transmitted by HBsAg negative blood containing anti-HBc. Med Lab Sci 1982; 39: 61–62.
9 Katchaki JN, Siem TH, Brouwer R. Serological evidence of presence of HBsAg undetectable by conventional radioimmunoassay in anti-HBc positive blood donors. J Clin Path 1978; 31: 837–839.
10 Tabor E, Hoofnagle JH, Smallwood LA, et al. Studies of donors who transmit post-transfusion hepatitis. Transfusion 1979; 19: 725–731.
11 Rakela J, Mosley JW, Aach RD, et al. Viral hepatitis after transfusion with blood containing antibody to hepatitis B core antigen. Gastroenterology 1980; 78: 1.318.
12 Nath N, Fang CT, Dodd RY. A simple technique for en-

hancing the sensitivity of a commercial solid-phase radioimmunoassay for hepatitis N surface antigen (HBsAg). Transfusion 1981; 21: 457–461.
13 Ben Porath E, Wands J, Gruia M, Isselbacher K. Clinical significance of enhanced detection of HBsAg by a monoclonal radioimmunoassay. Hepatology 1984; 4: 803–807.
14 Ben Porath E, Wands JR, Bar-Sany S, Huggins C, Isselbacher K. Improved detection of hepatitis B surface antigen (HBsAg) in blood donors by monoclonal radioimmunoassay. Transfusion 1985; 25: 10–14.
15 Odell WD, Willber JF, Paul WE. Radioimmunoassay in human serum. J Clin Endocr Metab 1965; 25: 1179–1183.
16 Cossart YE, Kirsch S, Ismay SL. Post-transfusion hepatitis in Australia. Lancet 1982; i: 208–213.
17 Archer AC, Cohen BJ, Mortimer PP. The value of screening blood donors for antibody to hepatitis B core antigen. J Clin Path 1983; 36: 924–928.
18 Peláez S, Sánchez-Quijano A, Lissen E, et al. Prevalencia de anti-HBc e hipertransaminasemia asintomática en donantes voluntarios HBsAg negativo de Sevilla. Gastroenterol Hepatol 1983; 6: 533–536.
19 Acero D, Profitós J, Martí MJ, Palomer J, Caballería J, Bruguera M. Anti-HBc y hepatitis B postransfusional. Problemas pendientes de solución. Med Clin (Barc) 1984; 82: 910–911.
20 Roggendorf M. Significación clínica de los diferentes marcadores de riesgo de la hepatitis B. In: R Vázquez, F García de Pesquera, C Herrera, D García (Eds.), Serología de las Hepatitis Viricas. Gráficas Rublán, Sevilla, 1982: 37–50.
21 Roggendorf M, Deinhardt F, Frösner GG, Scheid R, Bayerl B, Zachoval R. Immunoglobulin M antibodies to hepatitis B core antigen: evaluation of enzyme immunoassay for diagnosis of hepatitis B virus infection. J Clin Microbiol 1981; 13: 618–626.
22 Kojima M, Kazumichi U, Takahashi Y, et al. Correlation between titer of antibody to hepatitis B core antigen and presence of viral antigens in the liver. Gastroenterology 1977; 73: 664–667.
23 Harris RB, Semar M, Johnson AJ. Detection of hepatitis B surface antigen in potentially contaminated human plasma and plasma fractions. J Lab Clin Med 1977; 80: 1107–1114.
24 Park H. New technique for solid-phase immunoassay: application to hepatitis B surface antigen. Clin Chem 1979; 25: 178–182.
25 Wands JR, Carlson RI, Shoemaker H, Iseelbacher KJ, Zurawski VR. Immunodiagnosis of hepatitis B with high-affinity IgM monoclonal antibodies. Proc Nat Acad Sci (USA) 1981; 78: 1214–1218.
26 Wands JR, Bruns RR, Carlson RI, Ware A, Menitove JE, Isselbacher JK. Monoclonal IgM radioimmunoassay for hepatitis B surface antigen: high binding activity in serum that is unreactive with conventional antibodies. Proc Nat Acad Sci (USA) 1982; 79: 5675–5679.
27 Shafritz DA, Lieberman HM, Isselbacher KJ, Wands JR. Monoclonal radioimmunoassay for hepatitis B surface antigen. Demonstration of hepatitis B virus DNA or related sequences in serum and viral epitopes in immune complexes. Proc Nat Acad Sci (USA) 1982; 79: 5675–5679.
28 Wands JR, Marcianiak RA, Isselbacher KJ, et al. Demonstration of previously undetected hepatitis B viral determinants in an Australian aboriginal population by monoclonal anti-HBs antibody radioimmunoassay. Lancet 1982; i: 977–980.
29 Wands JR, Fujita YK, Isselbacher KJ. Identification and transmission of hepatitis B virus (HBV) related variants. Hepatology 1985; 5: 1054.