Revista Mexicana de Patología Clínica

Volumen *Volume* **51** **Número** *Number* **3** **Julio-Septiembre** ***July-September*** **2004**

Artículo:

Low cost latex agglutination test (LAT) for the procurement of plasma containing antibodies against hepatitis B surface antigen

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Key words: Immunoglobulin anti-HBs, latex agglutination, human plasma.

Palabras clave: Inmunoglobulina anti-HBs, aglutinación látex, plasma humano.

Recibido: 27/12/2003
Aceptado: 16/04/2004

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Abstract

Objective: In this work, a sensitive and specific Latex Agglutination Test (LAT) for detection and screening of antibodies to Hepatitis B Virus (HBV) in human serum or plasma is described. **Methods and results:** This test revealed a good correlation with Enzymeimmunoassay (EIA), (r:0.91) and the interassay coefficient of variation was 8-15% showing an adequate reproducibility. The sensitivity was adjusted at levels of antibody around 0.1 UI/mL and the specificity was determined by a competitive assay. **Conclusion:** This procedure is inexpensive and very simple, and can be applied for the studies and large scale screening of blood donors in the immune Hepatitis B plasma procurement for the production of the respective hyperimmunoglobulin.

Resumen

Objetivos: En este trabajo, se describe un test de aglutinación látex específico y sencillo (LAT) destinado a la búsqueda y detección de anticuerpos contra el virus de la hepatitis B (VHB) en muestras de plasma humano destinado a la producción de la inmunoglobulina específica. **Métodos y resultados:** El test reveló tener una buena correlación con enzimoinmunoensayo (EIA), (r:0.91) y el coeficiente de variación interensayo se encontró entre 8-15% demostrando una reproducibilidad adecuada para nuestros propósitos. La sensibilidad fue ajustada a niveles de anticuerpo iguales a 0.1 UI/mL y la especificidad fue determinada por ensayos competitivos. **Conclusión:** El procedimiento descrito resulta ser de bajo costo y de fácil aplicación, por lo que puede ser aplicado en el estudio y en la búsqueda poblacional de donantes de sangre para la obtención de plasma hiperinmune de la región, destinado a la producción de una inmunoglobulina contra el virus de la hepatitis B.

Introduction

The administration of hyperimmune Hepatitis B immunoglobulin (HBIG) is an effective media for providing passive immunization in preventive cases to individuals exposed to Hepatitis B Virus (HBV).[1-4,8,14]

In spite of the existence of a safe vaccine directed to the generation of an active immunization against HBV, HBIG is still applied in situations that involve needle-snick accidents or perinatal exposure to blood or body fluids infected with HBV.[6-9]

According to the above, HBG is an useful plasma derivate whose production requires of a correct supply of human plasma containing high levels of anti-HBs; so, the procurement of anti-HBs hyperimmune plasma by means of large scale screening of blood donors requires to apply an adequate technique. In relation to it, several methodologies as counterimmunoelectrophoresis (CIEF), enzymeimmunoassay (EIA), and radioimmunoassay (RIA) have been described.[1,2,4,5,10,14]

This study describes a low cost latex agglutination test that is principally adaptable to developing countries, directed to the selection of plasma with an anti-HBs level higher than 1 UI/mL.[3,9,20]

Material, methods and subjects

Obtention of HBsAg to coat latex particles

We applied a technique which combines the methods described by Gerin and De Rizzo.[16,17]

Briefly, a pool of HBsAg positive serum was heated at 60ºC for 18 h and HBsAg was precipitated with 11% PEG-6,000 solution. The sediment was redisolved in PBS (0.1 N, pH 7.2) and submitted to ultracentrifugation in cesium chloride gradient. The final product obtained from ultracentrifugation rendered a solution of HBsAg with a concentration of 620 ng/mL and a specific activity of 2.60 ng/μg.

Latex particles sensitization

Sensitized latex particles were prepared as follow: one volume of 1:5 dilution of stock particles in PBS, was mixed with one volume of HBsAg solution (620 ng/mL) previously diluted 1:2 with borate buffer pH 9.6. Following the incubation at 37ºC for 30 min, the particles were centrifuged and the final pellet was washed three times with bidestilled water, finally it was resuspended at 2% in PBS and stored at 4ºC until to be used.[15,19,21]

The optimum concentration of purified HBsAg solution for coating was determined assaying particles that were sensitized with different dilutions of antigen and evaluated against a reference HBIG (CLB, Central Laboratory of the Netherlands Red Cross Amsterdam, the Netherlands).

Agglutination test

An aliquot of 25 μL of 2% latex sensitized particle suspension was added to an equal volume of serum. After allowing the mixture to stand at room temperature during 15 min, the results were determined by view lecture and evaluated according to a semi quantitative scale.

HBsAg and Anti-HBs quantitation

HBsAg and anti-HBs determinations were carried out by using commercial reagents (Abbott Laboratories, North Chicago, Illinois, USA). Determination of anti-HBs activity.

The anti-HBs activity present in a standard preparation was determined by EIA and this method was used as a reference to compare the results obtained by LAT. The EIA reference curve was performed as follow:

Ten-twofold dilutions were made from a 100 IU/mL reference HBIG (CLB) and all dilutions were assayed by AUSAB (Abbott) including a quantitation panel. The anti-HBs content of the serum sam-

ples was determinate using the linear part of the reference HBIG curve.[12]

Subjects

One thousand and three hundred twenty blood samples were obtained from voluntary non-remunerated donors in the age group between 18 and 50 years, from the Cordoba National University Blood Bank.[18]

Statistical analysis

Analysis of correlation between LAT and EIA was performed applying no parametric statistical methods.[19]

Results

Assessment of assay characteristics

The sensitivity of the assay was evaluated with different dilutions of a reference HBIG whose reaction grades were considered as a standard agglutination pattern that was used as a reference control *(table I)*. The agglutination degree (established in +) for several human serum samples with different anti-HBs levels and standard HBIG is showed in *table II*, revealing that samples containing anti-HBs levels around or higher than 0.1 UI/mL were detected.

Table I. Agglutination grade of the LAT with different dilutions of reference HBIG.

Anti-HBs (IU/mL)*	Dilution factor**	Agglutination Grade
0.10	1/8	+
1.00	1/32	+ +
0.93	1/32	+ +
0.98	1/32	+ +
4.60	1/64	+ + +
6.80	1/128	+ + + +
7.00	1/128	+ + + +
10.00	1/256	+ + + + +
100.00	1/1,024	+ + + + + +
Negative	—	—

* Determinated by EIA ** Determinated by LAT

Table II. Agglutination grade of the LAT assayed against different human anti-HBs (+) sera.

Sample	Anti-HBs (IU/mL)	Agglutination grade
1	0.210	+
2	0.053	-
3	0.205	+
4	0.928	+ +
5	0.976	+ +
6	0.094	+
7	7.000	+ + + +
8	7.000	+ + + +
9	8.000	+ + + +
10	10.000	+ + + + +
HBIG*	1.000	+ +
HBIG*	10.000	+ + + + +
Negative 1	—	—
Negative 2	—	—
Negative 3	—	—

* Standard HBIG
– 1 to 10: human serum samples.
– Negative: human serum sample without anti-HBs.

In order to insure that sensitized latex particles were indeed detecting anti-HBs, a serum sample containing high HbsAg concentration (56 ng/mL) was serially diluted and each dilution was mixed with an anti-HBs reference serum (1 UI/mL), prior to the addition of the sensitized particles.

Furthermore, its should be stated in material & methods the origin of this high HBsAg concentration sample and which methodology was used to determine its concentration, when a sample of 1 UI/mL is previously incubated with high concentrations of HBsAg, the agglutination observed disappears. Also, the specificity was confirmed assaying the sensitized latex particles front 50 samples of sera pools containing other antibodies. The samples were: 10 anti-Tetanus, 5 anti Rubella, 6 anti HAV (Hepatitis A Virus), 4 anti-D (Rho), and with an anti-HBs positive control from a pool of 25 se-

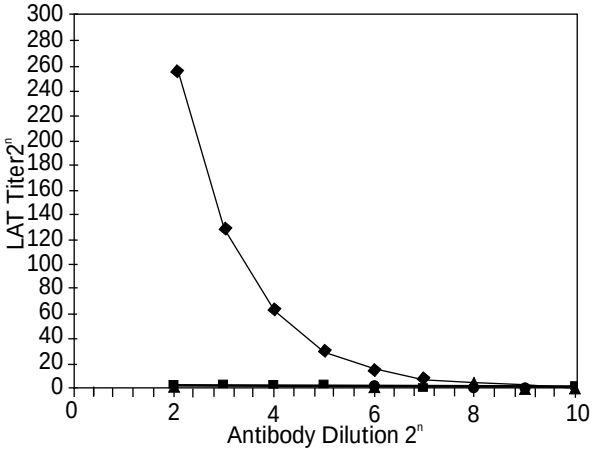

Figure 1. Specificity of the LAT. Assay of agglutination from sera containing individually Anti Tetanus■, Anti HAV▲, HNS●, anti-HBs◆, HNS: Human Normal Serum

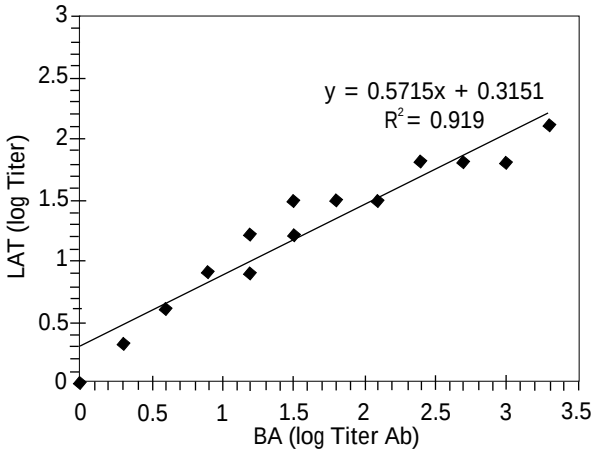

Figure 2. Correlation assay (LAT *vs* EIA).

rum samples; only with the dilutions of the serum containing specific antibody, positive reactions were observed *(figure 1)*.

To assess the reproducibility of the LAT, the assay was carried out on the same serums samples (n = 3) on five times simultaneously (intra assay), and at different days (inter assay); no differences in the results were detected intra assay *(table III)*. *Table IV* reveals a good reproducibility inter assay of LAT, indicated by the coefficient of variation on three representatives samples (8.1-15.7%).

In the *figure 2*, the correlation between LAT and EIA, considered as a reference method, is showed; the regression curve presents a positive intersection and a lightly negative pendent. The F and t values were 2.7 and 0.79, respectively (the critical values for 8 freedom degree are F:3.35 and t:1.73) for p = 0.05.

Table III. LAT: Simultaneous repeability assay.

Assay	Sample 1	2	3
1º Time	1:32	1:64	1:512
2º Time	1:32	1:32	1:512
3º Time	1:16/1:32	1:64	1:512
4º Time	1:32	1:128	1:512
5º Time	1:32	1:64	1:512
Media titer	1:32	1:64	1:512

The effect of reaction temperature and incubation time on LAT

The impact of reaction temperature was evaluated employing positive and negative controls and six positive serum that were assayed at several temperatures (5º, 15º, 25º, 30º, 37ºC), and no differences were observed at the temperature range assayed *(table V)*.

To evaluate the relevance of incubation time, dilutions of positive and negative serum samples at different dilutions were analyzed incubating sensitized particles with the samples at time between 3 and 30 min. Similar results were observed in the range of 10-30 min *(table VI)*, and according to it 15 min was considered as a practical time to perform the LAT.

Stability study

Samples of sensitized latex particles were stored at –18ºC, 4ºC and 25ºC (room temperature) dur-

Table IV. Reproducibility of the LAT: the same sample assayed in five different days. The results are expressed like a Log_{10} titer media.

Sample number	Number of observations (each one performed in 5 different days)	Log_{10} titer X ± SD	CV %
1	13	0.66 ± 0.10	15
2	15	1.10 ± 0.17	15.7
3	15	0.86 ± 0.07	8.1

Table V. LAT: Effect of the reaction temperature.

Sample number	Reaction temperature				
	5º C	15º C	25º C	30º C	37º C
1	1:1,024	1:1,024	1:1,024	1:1,024	1:1,024
2	1:1,024	1:1,024	1:1,024	1:1,024	1:1,024
3	1:256	1:256	1:256	1:256	1:256
4	1:128	1:64	1:64	1:64	1:32
5	1:64	1:64	1:64	1:64	1:16
6	1:32	1:32	1:32	1:32	1:8
Positive	1:32	1:32	1:32	1:32	1:8
Negative	Neg.	Neg.	Neg.	Neg.	Neg.

Table VI. LAT: Effect of the incubation time.

Time (min)	Sample		
	1	2	3
3	Neg.	Neg.	Neg.
5	1:32	1:8	Neg.
10	1:32	1:8	Neg.
15	1:32	1:8	Neg.
20	1:32	1:8	Neg.
30	1:32	1:8	Neg.

ing different times: 30, 60, 120 and 180 days and than the test was evaluated.

In all cases, the best performance was obtained when the particles remained at room temperature (25ºC) *(figure 3)*.

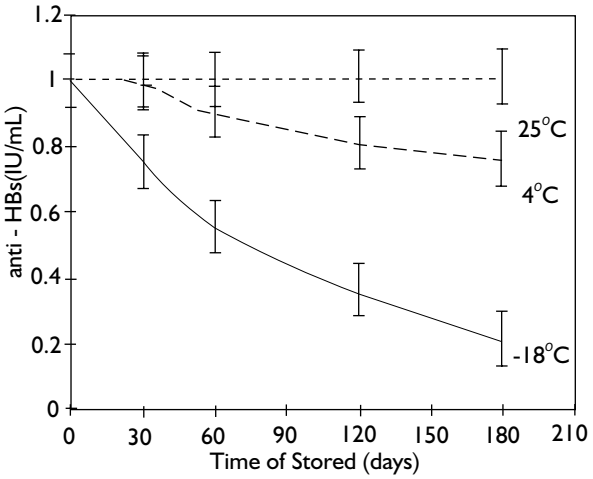

Figure 3. Stability of the sensitized particles stored at different temperatures during 180 days, agglutination assay at different times of stored.

Determination of cut-off

The interpretation pattern was established by comparing the results obtained with 135 serum samples (55 positive and 80 negative) tested by LAT and EIA. A dilution 1:32 of HBIG standard (CLB) determined by LAT, corresponded with an anti-HBs level of 1 IU/mL evaluated by EIA. Considering that the minimal requirements of anti-HBs concentration in plasma directed to the production of HBIG is 1 IU/mL, agglutination was adjusted to give the positive/negative cut off point at 1 IU/mL.

Discussion

The availability of an screening method for the selection of blood donations containing any defined level of anti-HBs, has a relevant importance for plasma selection directed to the production of HBIG.

In general, Blood Banks and the Hemotherapy Centres are employing sensitive immunoassay techniques (EIA, RIA)[4,7] for the detection of HBsAb, but with a high cost for the laboratories of undeveloped and developing countries and are time and labour consuming.

Fritz and Rivers[20] described a latex agglutination test for detection of HBsAg and that additionally could be applied in the determination of HBsAb. In this work, we describe a rapid and low cost LAT for direct antiHBs detection in human serum or plasma.

LAT described in this work was designed to be used in the plasma procurement for the HBIG large-scale production and according to it the threshold of the assay was adjusted to select the level of antibody content, which will be accepted, to produce an immunoglobulin with a potency of at least 100 IU/mL, considering to the regulatory requirements of the European Pharmacopoeia for this hyperimmunoglobulin.[22]

Respect to the evaluation of LAT performance, the values of the coefficient of variation (8-15%) revealed an adequate reproducibility when a same sample was assayed on different days and showed a good correlation with EIA (r:0.91). The specificity was confirmed with a competitive assay in which a defined amount of anti-HBs from human origin could block the binding to latex sensitized particles of anti-HBs contained in a serum sample.

The sensitized latex particles have been found to be stable for at least 5 months at 25ºC, and it allows their use under not refrigerated conditions. Also from the practice viewpoint, LAT insumes a short time of analysis because can be completed in 15 minutes.

With the results observed in this work we considered that this LAT is exact, precise and reveals a good sensitivity for ours requirements.[24]

If HBsAg coated latex particles are prepared in the laboratory; the only significant additional reagent cost: is the pourchase of latex. The cost: is therefore substantially less than the current cost: of commercial EIA or RIA and not requires sophisticated equipment and trained personnel.

For these reasons, we believe that the LAT is very useful, and can be employed in developing countries like MERCOSUR community, for the large scale screening of blood donor in the Immune Hepatitis B plasma procurement applied to the production of regional hyperimmune anti-HBs globulin.

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