Detection of Hepatitis B Virus Large Surface Protein Using a Time-Resolved Immunofluorometric Assay

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Background: To establish a novel method based on time-resolved immunofluorometric assay (TR-IFMA) with higher sensitivity and a broader detection range for detecting serum hepatitis B virus large surface protein (L protein). **Methods:** The precision, sensitivity, specificity, coefficient of recovery, and stability of the assay were evaluated and comparison with the classical enzyme-linked immunosorbent assay (ELISA) was also executed. **Results:** The precision, specificity, and sensitivity of the TR-IFMA were clearly better than ELISA. Particularly, the sensitivity was 0.1 ng/ml; moreover, the specificity was 100%, 96%, 92.5%, 96.9%, 97.8%, and 100% in the sera of healthy blood donors, systemic lupus erythematosus (SLE) patients, rheumatoid arthritis (RA) patients, hepatitis C virus (HCV) patients, cytomegalovirus (CMV) infection patients, and pregnant patients, re-

spectively. Meanwhile, we observed that the established TR-IFMA kit has a wider acceptable linear range of 0.63–10,367 ng/ml rather than the regular commercial ELISA kit having range of only 10.12–1095.9 ng/ml. Subsequently, correlation coefficient between the TR-IFMA and ELISA was 0.8009. The intra- and interassay precision rates were less than 5% for three different concentrations. The average recovery rate for L protein was 101.17%. In sum, the established assay kit performed better in terms of stability than the commercial ELISA kit. **Conclusion:** The TR-IFMA that we developed for L protein presented a higher sensitivity and wider detecting range than regular commercial ELISA. Therefore, this TR-IFMA has promising value both in the screening of HBV and monitoring of antiviral therapy. J. Clin. Lab. Anal. 29:498–504, 2015. © 2014 Wiley Periodicals, Inc.

Key words: enzyme-linked immunosorbent assay; hepatitis B virus large surface protein; time-resolved immunofluorometric

INTRODUCTION

The hepatitis B virus (HBV) is the main human pathogen responsible for severe hepatic diseases such as cirrhosis and hepatocellular carcinoma. Even though infection can be prevented by immunization with an efficient vaccine, still about 2 billion people have been infected worldwide, resulting in 350 million chronic carriers who are prone to liver diseases (1). Therefore, effective monitoring of HBV infection is mandatory from a public health perspective, particularly in highly epidemic areas.

Generally, HBV surface antigen (HBsAg), HBV e antigen (HBeAg), HBV core antibody (HBcAb), and HBV DNA in serum have been used as standard markers of HBV infection and HBV replication. In some cases, HBV DNA test is performed in HBsAg-negative patients with

continuing chronic hepatic inflammation. The host's immune system strongly suppresses viral replication and gene expression of HBV, possibly aided by mutations in the surface antigen gene and precore region of the viral genome (2). On the other hand, examining the difference

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in HBsAg quantitation methods is needed for patients harboring other genotypes, namely E/F, that were hardly tested in the recent studies (3). HBeAg has been reported to be a reliable and sensitive marker of HBV replication (4). However, HBeAg-negative patients who were positive for HBeAg antibodies (anti-HBe) were reported to have chronic hepatitis B with replicating HBV (5), termed as HBeAg-negative chronic hepatitis B. At present, quantitation of serum HBV DNA is a crucial component in the evaluation of patients with chronic hepatitis B and in the assessment of HBV replication (6). However, the nucleic acid amplification test requires skilled operators and high costs, making it inappropriate for widespread usage in the developing countries.

The pre-S domains of the HBV large (L) surface protein fulfill two important functions in the viral life cycle on both sides of the viral envelope: binding to a virus receptor on the outside and to the nucleocapsid on the inside (7). The genome of HBV encodes two transcriptional activators: the HBx protein and PreS2-activator L protein. Both proteins trigger activation of c-Raf-1/MEK kinase cascade (8). Some studies indicate that the transcriptional activator function is crucial for HBV propagation (8–10). The data implicate that the L protein is a major cause of hepatocyte injury (11). In addition, the L protein is regarded as a new serum biomarker of HBV replication from chronic hepatitis B patients. A study (12) demonstrates that serum L protein is positive in some chronic hepatitis B patients (HBsAg- and/or HBeAg-negative) and could be used as a complementary marker with HBsAg and HBeAg.

A recently introduced technique for highly sensitive detection and quantitation of the L protein is the time-resolved immunofluorometric assay (TR-IFMA) that utilizes a TR fluorescence signal. The superiorities of this method include its log-fold dynamic range, low cost, flexibility, and that it does not require the use of hazardous materials such as radioactive iodine and cyanogen bromide (13). More attention has been given to the TR-IFMA that has been proved to be the most promising approach for the quantitation and detection of many diagnostic trace protein markers (14–17). The aim of this study was to establish a method of TR-IFMA with higher sensitivity and broader detection range for detecting serum L protein, and study the superiority of TR-IFMA over enzyme-linked immunosorbent assay (ELISA).

MATERIALS AND METHODS

Specimens

Sixty-six patients with chronic HBV infection were recruited from the Affiliated Wuxi People's Hospital of Nanjing Medical University. Patients were excluded if they

displayed any of the following: concomitant hepatitis C or D or HIV infection; autoimmune hepatitis, Wilson's disease, or primary biliary cirrhosis; a history of heavy alcohol intake or intake of any hepatotoxic agents. Thirty serum samples without HBV markers from noninfected individuals were used as controls.

All blood specimens were collected from patients and healthy controls enrolled at the Affiliated Wuxi People's Hospital of Nanjing Medical University. Blood samples were taken once at entry from each patient after an overnight fasting period. Serum sample was separated by centrifugation at $1,917 \times g$ for 10 min and stored at -70°C prior to analysis. HBV DNA and the marker of HBV infection (HBsAg, HBeAg, anti-HBeAg) in serum were detected, and basic liver function tests (glutamic-oxalacetic transaminase, glutamic-pyruvic transaminase, albumin, total bilirubin) were performed simultaneously.

Microwell Plate Preparation

The anti-human L protein monoclonal antibodies (anti-L protein mAbs; Hotgen Biotech Co., Ltd., Beijing, China) were used for the assays. The specificity of mAbs had been described already by Wang et al. [15]. Concentrated anti-L protein mAbs were diluted to a final concentration of $3.5 \mu\text{g/ml}$ with $50 \text{ mmol/l Na}_2\text{CO}_3\text{--NaHCO}_3$ (pH 9.6) buffer (also used as coating buffer). A total of $200 \mu\text{l}$ of diluted anti-L protein was added to each well of MaxiSorp 96-well polystyrene microtiter plate (Nunc, Roskilde, Denmark) and incubated overnight at 4°C . Some binding sites not occupied by the coating buffer were then blocked by $250 \mu\text{l}$ of 2 g/l bovine serum albumin (BSA; Department of Health, Institute of Biological Products, Shanghai, China) in PBS (pH 7.4) and the plates were incubated overnight at 4°C . After the blocking buffer was discarded, the plates were vacuum dried and stored at -20°C until analysis.

Europium³⁺-Conjugation of Antibodies

Europium³⁺ (Eu^{3+}) labeling of antibodies was performed with the respective labeling kits (PerkinElmer Life Sciences, Wallac, Turku, Finland) according to the manufacturer's instructions. For the Eu^{3+} -anti-HBs polyclonal antibodies (anti-HBs Abs) preparation, 1 ml of anti-HBs Abs (2 g/l dissolved in 50 mmol/l PBS, pH 7.0) were loaded on a PD-10 column and eluted using carbonate buffer ($\text{Na}_2\text{CO}_3\text{--NaHCO}_3$, pH 8.5) containing 0.155 mol/l NaCl. Next, 2 g/l anti-HBs Abs were mixed with 0.2 mg Eu^{3+} -diethylenetriamine tetraacetic acid (DTPA; Sigma Aldrich, St. Louis, MO) followed by vigorous stirring at 30°C for 20 h. The resulting mixture was fractionated on a Sepharose CL-6B with 80 mmol/l Tris-HCl (pH 7.8) buffer. The absorbance of the eluate was

measured at 280 nm to obtain protein concentrations, and the labeling efficacy (Eu^{3+} mol/IgG mol) was measured. After purification, aliquots of the labeled antibody were stored at -20°C until use.

TR-IFMA Procedure

To measure the levels of serum L protein of HBV patients, a sandwich TR-IFMA was performed. When the L protein standard (640 ng/ml) was four times the serial dilution with 4% BSA in PBS (pH 7.4), 100 μl of L protein standards (2.5, 10, 40, 160, and 640 ng/ml; Hotgen Biotech Co., Ltd.), positive control (L protein positive serum), negative control (L protein negative serum), and samples were pipetted into each well and incubated with shaking (300 times/min) for 1 h at ambient temperature. The plates were washed four times with wash buffer (50 mmol/l Tris-HCl, 0.9% NaCl, 0.05% sodium azide, pH 7.4 with 0.01% Tween 20) and 100 μl of 206 nmol/l Eu^{3+} -anti-HBs Abs (1:20, dilution buffer was 0.05 mol/l Tris-HCl, pH 7.8, containing 0.9% NaCl, 0.2% of purified BSA, 0.01% Tween 20, 20 $\mu\text{mol/l}$ DTPA, and 0.05% sodium azide as preservative) were added to each well and incubated with shaking for 1 h at ambient temperature. The plates were washed six times, 200 μl of enhancement solution (PerkinElmer Life Sciences) was pipetted into each well, they were shaken for 5 min, and Eu^{3+} fluorescence intensity (cycles per second, cps) was measured with an auto DELFIA1235 TR-IFMA analyzer (PerkinElmer Life Sciences). The concentrations of the L protein in respective samples were determined based on the calibration curves.

L Protein Detection by ELISA

To measure the levels of serum L protein in HBV patients, two commercial ELISA kits (Hotgen and Beier, Beijing, China) were used. Both the kits were two-step sandwich ELISA kit. A total of 100 μl of L protein standards (2.5, 10, 40, 160, and 640 ng/ml) and samples were pipetted into each well of microplate coated by anti-L protein mAbs (Hotgen Biotech Co., Ltd.). The microplate was incubated for 1 h at 37°C . The plate was washed five times with PBS containing 100 μl of 0.2% Tween-20 (pH 7.4). Then, anti-HBs Abs conjugated to HRP were added to each well and incubated for 1 h at 37°C . The plate was washed five times, followed by the addition of 100 μl of substrate solution. The microplate was incubated at 37°C for 15 min in the dark. Then, 50 μl of stop solution was added and the absorbance at 450 nm was detected. The concentrations of samples were calculated from the standard curve.

HBV DNA Quantitation

Serum HBV DNA was quantified according to HBV DNA RT-PCR kits (Fuxing Pharmaceutical Co., Ltd., Shanghai, China). Briefly, DNA was extracted from serum using standard methods, including digestion with proteinase K, extraction with phenol/chloroform, and precipitation in cold pure ethanol. DNA was resuspended in distilled water and its concentration was determined by measuring the absorbance at 260 nm. PCR amplification of HBV DNA was carried out using a Lightcycler (Roche, Basel, Switzerland). The detection range was $420\text{--}4.2 \times 10^8$ IU/ml of serum. The results were expressed as HBV DNA IU/ml of serum.

Statistical Methods

Statistical analysis was performed using the SPSS software (SPSS, Inc., Chicago, IL). The L protein positive rates were compared between different patient groups by TR-IFMA and ELISA using the chi-square test (χ^2 test). Data followed a normal Gaussian distribution. Simple linear regression analyses were applied to determine the correlations between TR-IFMA and ELISA. Probability (P) values of <0.01 were considered to be statistically significant. Intra- and interassay variations were calculated using the coefficient of variation (CV).

RESULTS

Standard Curves

As shown in Figure 1, the TR fluorescence intensities are directly proportional to the concentrations of the L protein. The standard curve of fluorescent intensity versus the L protein was found to be linear over this concentration range with a correlation coefficient of 0.999. The linear equation for the calibration curve of the L protein was $\log(\text{cps}) = 3.433 + 0.9291 \log(\text{conc})$, where x is fluorescence intensity and y is the concentration of the L protein (ng/ml).

Cut-off Value

Normal reference ranges were established at 0–1.36 ng/ml based on values obtained from the 30 healthy volunteers. With the established immunoassay of serum (TR-IFMA), the mean ($\bar{x}$) is 0.45, standard deviation (SD) is 0.39, and the upper limit (cut-off value) is ($\bar{x} + 2.33 \times \text{SD} = 1.36$), with 99% unilateral paired t -test.

Sensitivity Testing

The sensitivity of the assay, in other words, the minimum detectable dose, was assessed by running zero

Fig. 1. Standard curves for HBV-LP by TR-IFMA at different concentrations (2.5, 10, 40, 160, and 640 ng/ml). The time-resolved fluorescence intensities are directly proportional to the concentrations of HBV-LP. Space between standard curve concentrations is log-transformed. The equation for the curve is $\log(\text{cps}) = 3.433 + 0.9291 \log(\text{conc})$, correlation coefficient = 0.999.

standard repeatedly ($n = 20$) within a single run. The mean adding two standard deviations of zero standards was considered as the minimum detectable dose, in other words, the sensitivity. The sensitivity of the TR-IFMA for the L protein was 0.1 ng/ml.

Specificity

The specificity of L protein detection was evaluated in the serum from followed groups including, 30 healthy blood donors, 25 SLE patients, 27 RA patients, 32 HCV patients, 45 CMV infection patients, and 26 pregnant patients recruited from noninfected HBV individuals and confirmed to be free from HBV markers of infection. The outcome was 100%, 96%, 92.5%, 96.9%, 97.8%, and 100% in the sera, respectively.

Linearity

Serum from patient with the high concentration of L protein (10,367 ng/ml) was diluted in a twofold serial dilution manner to 0.63 ng/ml. Then, serum and diluted sera were detected with the established TR-IFMA and two commercial ELISA kits (Hotgen and Beier). The linear curve of dilution concentration was obtained with dilution multiple as the horizontal abscissa and fluorescent intensity or absorbance as the vertical ordinate (Fig. 2). From this, the researchers observed that for the established TR-IFMA kit, there was an acceptable linear range within 0.63–10,367 ng/ml, whereas it was within 10.12–1,095.9 ng/ml for commercially available L protein assays,

Fig. 2. Serum from patient with the high concentration of HBV-LP (10,367 ng/ml) was diluted in a twofold serial dilution manner to 0.63 ng/ml. Serum and diluted sera were tested with TR-IFMA and ELISA (ELISA1—Hotgen and ELISA2—Beier), respectively. Double y-axis curve was determined according to dilution strength (x-axis) as well as TR-IFMA fluorescence intensity and ELISA absorbance (the two y-axes).

Fig. 3. Correlation analysis of HBV-LP tested between the TR-IFMA and ELISA in 66 samples with HBV $> 10^3$ IU/ml. Correlation coefficient between the TR-IFMA and ELISA was 0.8009.

indicating that the TR-IFMA has a wider detectable range than the commercial ELISA kits.

The Relationship Between the TR-IFMA and ELISA

The serum L protein concentration was tested in 66 samples with HBV $> 10^3$ IU/ml between the TR-IFMA and ELISA. Figure 3 shows a strong correlation between TR-IFMA and ELISA. Correlation coefficient was 0.8009.

Precision Testing

Serum samples with elevated L protein concentrations were diluted to high, intermediate, and low concentrations defined by ELISA quantitative kit results. Intrabatch

TABLE 1. Intra- and Interassay Precision Analysis in High, Intermediate, and Low Concentrations Defined by TR-IFMA Quantitative Kit

Group	Concentration (ng/ml) ^a	SD	CV (%) ^b
Intrabatch, <i>n</i> = 20			
High	357.6	8.78	2.46
Intermediate	53.4	1.57	2.94
Low	6.5	0.27	4.15
Interbatch <i>n</i> = 8			
High	358.7	11.25	3.13
Intermediate	53.6	1.97	3.68
Low	6.7	0.31	4.63

^aData are reported as the median.^bThe acceptance criteria of intra- and interassay precision rate were less than 5% (CV < 5%).**TABLE 2. Comparison of HBV-L protein Positive Rates Between TR-IFMA and ELISA**

TRFIA	ELISA		Total
	+	–	
+	51	8	59
–	0	7	7
Total	51	15	66

 $\chi^2 = 6.13, p < 0.01$.

variation was then defined at high, intermediate, and low concentrations by TR-IFMA for 20 times in one experiment. The intra-assay CVs of the L protein assay of three L protein levels were 2.46%, 2.94%, and 4.15%. Interbatch variation was determined by comparing values obtained from the analysis of the three sample concentrations tested by TR-IFMA for eight times on the basis of time-dependent experiments. The interassay CVs of the L protein assay of three L protein levels were 3.13%, 3.68%, and 4.63%. The acceptance criteria of intra- and interassay precision rates were less than 5% (CV < 5%) for three different concentrations. The TR-IFMA system showed good precision (Table 1).

Comparison of the L Protein Positive Rates by TR-IFMA or ELISA

Sixty-six samples with HBV DNA concentrations $> 10^3$ IU/ml were tested concurrently by TR-IFMA and ELISA ($\chi^2 = 6.13, P < 0.01$, Table 2). Among these, the L protein was detected positive in 51 samples by TR-IFMA and ELISA. However, eight samples were detected positive by TR-IFMA, which was negative by ELISA. The L protein was negative in the rest of seven samples detected by TR-IFMA and ELISA. The TR-IFMA system could test L protein concentration that ELISA assay could not detect.

TABLE 3. Coefficient of Recovery Analysis

Sample	Dilution	Observed (ng/ml)	Expected (ng/ml)	O/E (%) ^a
1		362.94		
	1:2	176.30	181.47	97.15
	1:4	95.35	90.74	105.09
2	1:8	43.52	45.37	95.93
		362.94		
	1:2	149.82	155.25	96.50
3	1:4	83.51	77.63	107.58
	1:8	37.62	38.81	96.93
		394.59		
	1:2	207.83	197.29	105.34
	1:4	106.16	98.65	107.62
	1:8	48.51	49.32	98.35

^aObserved concentrations $\times$ 100/expected concentration.

Analytical Recovery

Three sera with high L protein concentrations were diluted with sample buffer to three concentrations, respectively, and assayed in the new TR-IFMA kit. The recoveries of the first sample were 97.15%, 105.09%, and 95.93%. The recoveries of the second sample were 96.50%, 107.58%, and 96.93%. The recoveries of the third sample were 105.34%, 107.62%, and 98.35%. Average recovery rate (calculated average of the nine individual values by the formula: observed concentrations $\times$ 100/expected concentration) was 101.17% (Table 3). The acceptable criterion of recovery rate was 80–120%.

Stability of the TR-IFMA Kits

The assay kits were stored at 4°C for 7 days and at 37°C in water bath for 7 days. Then, the difference of fluorescence intensity or absorbance, that is, decline rate of combination, was measured between both by TR-IFMA and ELISA. It exhibited no visible change except for a decline by 14.25% in the rate of combination (27.54% and 25.34% for the commercial ELISA kits; Table 4). It suggested that the established assay kit had good stability and could be stored at 4°C for 1 year.

DISCUSSION

The L protein gains a complex transmembrane topology upon translation at the endoplasmic reticulum (18–20). It plays a crucial role in the targeting of HBV to liver cells, interaction with the core, and release of viral particles (21, 22). Serum L protein has been considered a novel marker of HBV infection, indicating viral replication and the presence of infectious virions (23). Both Chisari et al. (24) and Fernandes et al. (25) showed that transgenic mice with hepatocyte overexpression of the L protein had profound hepatocyte apoptosis and chronic

TABLE 4. Stability of the TR-IFMA and ELISAs

Groups	Preserved at 4°C for 7 days	Preserved at 37°C in water bath for 7 days	The declined rate of combination (%) ^c
ELISA1—Hotgen (abs) ^a	2.36	1.71	27.54
ELISA2—Beier (abs) ^a	2.21	1.65	25.34
TR-IFMA (cps) ^b	2,314,573	1,984,746	14.25

^aThe ELISA kits of Hotgen and Beier were detected according to absorbance (abs).

^bThe TR-IFMA kit was detected according to fluorescence intensity (cps).

^cThe declined rate of combination (%) = $(4^{\circ}\text{C (abs/cps)} - 37^{\circ}\text{C (abs/cps)}) / 4^{\circ}\text{C (abs/cps)}$.

hepatitis. They showed that the L protein plays a crucial role in the pathogenesis of HBV-related hepatitis. Wang et al. (12) offered a promising approach of ELISA for the diagnosis and treatment monitoring of HBV patients. They found a strong correlation between the levels of serum L protein and HBV DNA, which further demonstrated that L protein could be a candidate biomarker for assessment of HBV infection.

In previous studies, the L protein levels were measured with an ELISA (12, 26). However, this method does not provide enough sensitivity to detect the L protein of low concentration (27). This study proved that the detection limit and sensitivity of TR-IFMA are higher than those of ELISA (27, 28), and TR-IFMA has stronger early diagnostic potential for HBV infection. As a novel technology rapidly developed in the recent years, TR-IFMA, with characteristics of wide detection range, high sensitivity, easy automation, and less susceptibility to matrix interference, has received great attention. The TR-IFMA technology has been successfully used for quantitation and detection of analytes of clinical and biological value (17, 29, 30).

In this study, the sensitivity of the immunoassay for the L protein detection based on a classical sandwich TR-IFMA is 0.1 ng/ml, which is far lower than the minimum detectable dose of the ELISA for the L protein (5 ng/ml) from testing data of Wang et al. (12), indicating that it is sensitive enough to classify patients correctly as the L protein positive. In addition, the specificity (100%) of the L protein detection was evaluated by testing serum from 30 healthy individuals. No positive results from this population were observed. For the established TR-IFMA kit, there was an acceptable linear range within 0.63–10,367 ng/ml; inversely it was within 10.12–1,095.9 ng/ml for commercially available L pro-

tein assays, indicating that the TR-IFMA has a broader detectable range than the commercial ELISA. Moreover, the precision test, which is a major concern for an assay kit, also demonstrated that the TR-IFMA was better than the commercial ELISA, as its intra- and interassay CVs of the mixed serum specimens with high, intermediate, and low concentrations are all smaller than the latter. In addition, it was noted that the counts of fluorescent intensity obtained by the L protein TR-IFMA kit varied little, even when the microplates were stored overnight after the addition of enhance solution. However, the optical density obtained by the commercial ELISA kits decreased with time and could hardly put through 30 min after the addition of stop solution. This superiority in stability of TR-IFMA may largely be due to its independence of the disintegration of the labeling compound.

In summary, the TR-IFMA is a very useful tool for the L protein measurements in human serum specimens. The experimental results indicated that the new TR-IFMA is a highly sensitive and specific immunofluorescent assay; the assay also provides wider dynamic working ranges and better reproducibility. These findings may have potential value for the screening of HBV and monitoring of antiviral therapy.

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