Cost-Effective In-House Neutralization Assay for the Confirmation of HBeAg

Gnanadurai John Fletcher, Raghavendran Anantharam, Kalaivani Radhakrishnan, Amaldev Karunakaran, and Priya Abraham*

Department of Clinical Virology, Christian Medical College, Vellore, India

Background & Aim: Hepatitis B virus-e-antigen (HBeAg) is an affordable viral marker to assess viral replication kinetics and response to antiviral therapy. In the absence of confirmatory assays, discrepant or false-positive HBeAg results are resolved by screening for other HBV markers. We standardized an in-house HBeAg neutralization assay (HBeAg-NT) to confirm HBeAg in clinical samples. **Methods:** The performance and reliability of this assay were evaluated by first WHO International Standard for HBeAg (first WHO-IS HBeAg) from Paul Ehrlich

Institute and clinical samples ($n = 150$) from chronic HBV carriers. Of these, 71 HBeAg-positive sera were used for HBeAg-NT. **Results:** Concentrations spanning 0.25–10 U of first WHO-IS HBeAg and clinical samples (S/Co ranges from 1.00 to 10.00) were neutralized completely in the HBeAg-NT. **Conclusions:** HBeAg-NT is a simple, cost-effective, and reliable direct approach to confirm HBeAg in clinical samples which precludes the need for screening additional HBV markers in low resource settings. *J. Clin. Lab. Anal.* **30**:1146–1149, 2016. © 2016 Wiley Periodicals, Inc.

Key words: cost effective; HBeAg; in-house neutralization assay

INTRODUCTION

Worldwide, approximately 350–400 million people are infected with HBV. Chronic HBV (CHBV) infection and its fatal sequelae cause significant health problems (1). India harbors about 40 million CHBV carriers and the prevalence of HBV is 2.4% in general population and 15.9% among tribal populations (2).

HBeAg is one of the abundantly secreted multifunctional viral proteins of HBV. It has been used as an affordable viral marker to assess replication kinetics, treatment candidacy, and response to antiviral treatment. The presence of HBeAg in the serum indicates high viral replication and infectivity and it is also associated with high levels of HBV DNA. The persistence of HBeAg for >20 weeks suggests the progression to chronic HBV carrier phase. Furthermore, quantification of HBeAg has been shown to be highly valuable in assessing the efficacy of antiviral therapy (3,4).

Commercial HBeAg assays use various methods and principles to detect serum/plasma HBeAg. HBeAg detection is available in both automated immunoassay systems (AIS) and manual enzyme immunoassays (MIA). AIS have been shown to have substantial

differences in detection of HBeAg in clinical samples (5). In addition, certain AIS are prone to generate high rate of false-positive HBeAg results (6). According to WHO study, analytical sensitivity has been shown to vary significantly among HBeAg assays (7). Furthermore, the diagnosis of HBV infection is challenged by concomitant presence of HBeAg and anti-HBe. However, no confirmatory assays are currently available to confirm the presence of HBeAg in clinical samples. Furthermore, discrepancies in HBeAg detection are resolved by screening for other HBV markers. This indirect approach is not cost effective or time consuming and requires additional infrastructure. Hence, we attempted to standardize an enzyme immunoassay-based in-house HBeAg neutralization assay (HBeAg-NT) to directly confirm the presence of HBeAg in clinical samples.

*Correspondence to: Priya Abraham, Department of Clinical Virology, Christian Medical College, Vellore-632004, India. E-mail: priyaabraham@cmcvellore.ac.in

Received 9 March 2016; Accepted 17 April 2016

DOI 10.1002/jcla.21995

Published online in Wiley Online Library (wileyonlinelibrary.com).

MATERIALS AND METHODS

HBeAg Standard

Two vials of first WHO International Standard (IS) for HBeAg (100 IU/ml) were procured from Paul-Ehrlich-Institut (PEI), Germany. The IS was reconstituted and diluted using human plasma which was negative for HIV-1/2, anti-HCV, and all markers of HBV. Five serial dilutions were made to cover HBeAg concentrations spanning 0.125–10 IU/ml. As the analytical sensitivity of the HBeAg assay was 0.2 IU/ml, we have used three dilutions around this critical concentration and subjected to enzyme immunoassay-based in-house HBeAg neutralization assay (HBeAg-NT).

Clinical Samples

During the period of 2 years and 6 months (March 2013–September 2015), 3056 samples were tested for HBeAg. One hundred and fifty samples with adequate volume were selected to yield 79 HBeAg-negative samples and 71 HBeAg-positive samples. As low-positive HBeAg samples are prone to generate false-positive results, 71 low-positive samples were used for the neutralization assay. Consent was obtained from all patients and the study was approved by the Institutional Review Board of this Institution (IRB Min. No. 9470).

Serological Testing of HBV Markers

Detection of HBeAg

The sera of patients were tested for HBeAg in duplicate in ETI-EBK-PLUS (DiaSorin, Saluggia, Italy) assay or using Architect i2000SR (Abbott, Wiesbaden, Germany) as part of diagnostic protocol. ETI-EBK-PLUS is a direct, two-step “sandwich” enzyme immunoassay (EIA). Likewise, Architect HBeAg is a two-step CMIA and the detection is performed in a closed automated analyzer, Architect i2000SR (Abbott).

Detection of anti-HBe

Anti-HBe screening was done for patients who showed low (S/Co ratio 1–10) HBeAg reactivity in the initial screening assay (ETI-EBK-PLUS). Samples that were low positive for HBeAg in the initial testing were tested for anti-HBe by Architect anti-HBe assay (Abbott).

Standardization of an EIA-Based in-House HBeAg Neutralization Assay

Principle

Specific antibody (anti-HBe) was used as neutralization agent to confirm the presence of HBeAg in clinical samples. Pooled antibody to HBeAg (anti-HBe, human) was pre-incubated (37°C) with clinical sample along with dilution controls. If HBeAg was present in the serum, it would bind specifically to the anti-HBe. The HBeAg–anti-HBe complex proportionally blocked HBeAg from binding to the mouse monoclonal antibodies to HBeAg coated on the microwells. This resulted in reduction in optical density (O.D.) when compared to the dilution control and undiluted sample (neat). A specimen was confirmed positive, if the percent neutralization was $\geq 90\%$ or greater.

Preparation of neutralization reagent (anti-HBe)

Samples received routinely for HBV diagnosis and therapeutic monitoring from CHBV carriers were tested for antibodies to HBeAg (anti-HBe) by Architect anti-HBe (Abbott) which works on the competitive principle. Ten strongly reactive samples were pooled, centrifuged (1000 g for 10 min), and filtered (0.22 μ m, Millipore, UK). The reactivity of anti-HBe was checked again and the neutralization agent (anti-HBe) with adequate reactivity (Sample OD/cut-off value (S/Co) ≤ 0.05) was stored at -20°C . Furthermore, the neutralization potential of the reagent (anti-HBe) was checked against various concentrations of first WHO-IS HBeAg.

Procedure

Two hundred and fifty microliters of clarified patient serum was transferred into three tubes. Equal amount of pooled antibody to HBeAg (neutralizing reagent) was added into one tube and negative plasma (dilution control) into another tube. All three tubes were mixed adequately and centrifuged briefly at 500 g, and incubated at 37°C for 1 h. After incubation, tubes were centrifuged at 1000 g for 10 min and loaded in duplicate into the respective ELISA wells. HBeAg EIA (ETI-EBK-PLUS) was performed as per manufacturer instructions. Criteria of validation, grading, results, and interpretation of EIA-based in-house HBeAg confirmatory assay are shown in Table 1.

Automated washing was carried out in ELISA washer (ELx50; BioTek, Winooski, VT, USA) and OD was measured at dual wavelength (450/630 nm) in a spectrophotometer (Epoch/ μ Quant; BioTek). Manufacturers' instructions were followed strictly for all assays.

TABLE 1. Grading and Validation of In-House HBeAg Neutralization Assay

Criteria for HBeAg neutralization			Grading of HBeAg neutralization	
Tube 1	Tube 2	Tube 3		
Undiluted sample OD	Dilution control OD Sample + Negative control ^a	Neutralization OD Sample + anti-HBe	Calculation of % neutralization of HBeAg ^b	Results/interpretative comments
Neat				
≥ twice the COV to ≤1.000	>COV	<COV%	>90–100%	Low positive/HBeAg optimum for neutralization
>COV to <1.000	>COV	>COV	<50%	Negative/False positive
>COV	<COV	<COV	NA	HBeAg too low for reliable neutralization

^aNegative control is a serum of pooled sample negative for HBsAg, anti-HBc total, HBeAg, and anti-HBe.

^bFormula for the calculation of % neutralization of HBeAg $\frac{\text{OD of Tube 2} - \text{OD of Tube 3}}{\text{OD of Tube 2}} \times 100$.
OD, optical density; COV, cut-off value; NA, not applicable.

RESULTS

Sample Characteristics

During initial screening 79 patients were HBeAg negative and 71 were HBeAg positive in ETI-EBK-PLUS. All 71 samples were stratified as low positive based on S/Co ratio of 1.00 to ≤10.00.

Performance of In-House HBeAg Neutralization Assay

The neutralization reagent (pooled anti-HBe) used in this assay successfully neutralized all three concentrations (0.25, 0.5, and 10 IU/ml) of first WHO International HBeAg Standard (IS) (PEI) (Fig. 1).

Furthermore, samples ($n = 64$) that were low (S/Co ratio 1.00–10.00) HBeAg positive in ETI-EBK-PLUS were effectively neutralized (Fig. 2). After dilution, seven samples with very low HBeAg reactivity became negative and these samples were also low anti-HBe positive. In addition, five more samples showed low reactivity for anti-HBe. Samples that had adequate HBeAg concentration for neutralization showed no false-positive results.

DISCUSSION

HBeAg assays are prone to generate discrepant or false-positive results (5, 6). However, discrepancies in HBeAg detection are resolved currently by testing additional HBV markers. This indirect approach not

Fig. 1. Neutralization of various concentrations of first WHO- HBeAg International standard.

Fig. 2. Neutralization of various concentrations of HBeAg in clinical samples.

only increases the turnaround time but also escalates the cost of testing.

As EIA is most widely used and cost effective, EIA-based HBeAg-NT assay was standardized to confirm HBeAg in clinical samples. The principle, procedure, and interpretation of this assay are similar to in-house HBsAg neutralization assay developed earlier in the laboratory to confirm low HBsAg reactive samples (8).

The pooled anti-HBe used in the HBeAg-NT assay was able to successfully neutralize various concentrations of HBeAg in International Standard and in clinical samples. The neutralization agent (anti-HBe) with adequate titer and specificity can be obtained easily from CHBV carriers who have seroconverted into anti-HBe. Similar to HBsAg neutralization assay, HBeAg-NT can be used strategically to confirm low reactive samples (9). In addition, samples with S/Co of <2.00 cannot be confirmed reliably by the neutralization assay.

In conclusion, HBeAg-NT is a simple, cost effective, and reliable confirmatory assay to confirm HBeAg in clinical samples. This assay improves the overall quality of HBeAg testing, adds comprehensiveness, and precludes the need for additional testing of other HBV markers in low resource settings.

ACKNOWLEDGMENTS

This study was funded by Department of Clinical Virology (22F060), Christian Medical College, Vellore.

Sincere thanks to Dr. Hubert Darius Daniel (Consultant Virologist) for his valuable advice and suggestion.

REFERENCES

1. Maddrey WC. Hepatitis B: An important public health issue. *J Med Virol* 2000;61:362–366.
2. Batham A, Narula D, Toteja T, Sreenivas V, Puliye JM. Systematic review and meta-analysis of prevalence of hepatitis B in India. *Indian Pediatr* 2007;44:663–674.
3. Lee JM, Ahn SH, Kim HS, et al. Quantitative hepatitis B surface antigen and hepatitis B e antigen titers in prediction of treatment response to entecavir. *Hepatology* 2011;53:1486–1493.
4. Maylin S, Boyd A, Martinot-Peignoux M, et al. Quantification of hepatitis B e antigen between Elecsys HBeAg and Architect HBeAg assays among patients infected with hepatitis B virus. *J Clin Virol* 2013;56:306–311.
5. Kim H, Oh EJ, Kang MS, Kim SH, Park YJ. Comparison of the Abbott Architect i2000 assay, the Roche Modular Analytics E170 assay, and an immunoradiometric assay for serum hepatitis B virus markers. *Ann Clin Lab Sci* 2007;37:256–259.
6. Cheng L, Guan Q, Zhang J, Sun Z. Discrepancies between two automated immunoassay systems in determining hepatitis B virus markers in serum samples with concomitant presence of antigens and antibodies. *Ann Clin Lab Sci* 2010;40:49–52.
7. Annette R. Collaborative study to establish a World Health Organization international standard for Hepatitis B e antigen (HBeAg). World Health Organization; 2013; Available from: <http://www.who.int/iris/handle/10665/96313> Last accessed September 8, 2014
8. Fletcher GJ, Gnanamony M, David J, Ismail AM, Subramani T, Abraham P. Do we need an 'in-house' neutralization assay for confirmation of hepatitis B surface antigen? Answers from a tertiary care hospital in India. *J Gastroenterol Hepatol* 2010;25:942–945.
9. O'Brien JE. Hepatitis B surface antigen: Decreased need for confirmation of reactive results. *Clin Chem* 2000;46:582.