

HEPATOLOGY

Do we need an 'in-house' neutralization assay for confirmation of hepatitis B surface antigen? Answers from a tertiary care hospital in India

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Key words

anti-hepatitis B core antibody, enzyme immunoassay, hepatitis B surface antigen, hepatitis B virus.

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Abstract

Background and Aims: Hepatitis B surface antigen (HBsAg) is an important serological marker for diagnosis of hepatitis B virus (HBV) infection. Commercial kits for detection of HBsAg emphasize confirmation by neutralization assays. In this study, we have standardized an 'in-house' neutralization test for HBsAg confirmation.

Methods: Among 6684 HBsAg-positive samples, 615 were subjected to an 'in-house' HBsAg neutralization test (NT). Of these, 91 (100%) high-reactive samples (optical density [OD] 1.000–3.000) and 286 (93%) of 289 low-reactive samples (OD < 1.000) were neutralized, and 235 (100%) grey-zone reactive samples were 'in-house' NT negative. Eighty-four samples of varying reactivities that were tested by the 'in-house' NT were compared with a commercial NT (AxSYM, Abbott).

Results: The 'in-house' NT showed an excellent agreement ($\kappa = 0.83$, $P < 0.001$) with the commercial confirmatory assay. The sensitivity, specificity, positive and negative predictive values were 90%, 94%, 96% and 87%, respectively.

Conclusion: The enzyme immunoassay-based 'in-house' HBsAg neutralization assay is a feasible alternative to the commercial HBsAg confirmatory assay. This technique is easily adaptable, cost-effective and reliable for the confirmation of HBsAg in a low resource setting, enhancing the overall quality of HBsAg screening.

Introduction

Hepatitis B virus (HBV) infection and its fatal sequelae are major health problems.¹ An estimated 400 million persons worldwide are chronically infected with HBV.² The prevalence of HBV in the tribal and non-tribal population in India is 15.9% and 2.4%, respectively.³ Transmission of HBV occurs primarily through infected blood, sexual contact and the vertical route.⁴

Hepatitis B surface antigen (HBsAg) is the most widely tested marker to detect HBV infection. Detection of HBsAg in the peripheral blood is hence crucial for the identification of infected blood units and clinical samples. Accurate and timely detection impacts on the epidemiology and its fatal sequelae. Other HBV markers such as immunoglobulin (Ig)M anti-hepatitis B core antibodies (anti-HBc IgM), total anti-HBc (anti-HBc), anti-hepatitis B e-antibodies (anti-HBe) and anti-hepatitis B surface antibodies (anti-HBs) contribute significantly to the laboratory diagnosis of HBV infection. However, the detection of HBsAg in diagnostic virology is challenged by the occurrence of ambiguous reactivity, false positivity and logistic limitations of performing confirmatory tests. The cost of additional HBsAg confirmatory assays is extremely prohibitive. Hence, we standardized an 'in-house'

HBsAg neutralization assay in an attempt to reduce the cost of HBsAg confirmatory testing, yet ensuring the quality of HBsAg detection in the laboratory.

Methods**Subjects and study design**

During the period of January 2005 to December 2007, blood samples were received from 204 523 individuals for HBsAg screening from out-patient clinics and in-patient wards in the Christian Medical College (CMC), a tertiary care teaching hospital in Vellore, India. A general consent was obtained in this hospital for all investigations as part of routine patient management. To analyze a wide range of enzyme immunoassay (EIA) reactivities as measured by optical densities (OD), 91 high-reactive (OD 1.000–3.000) samples, 289 low-reactive (OD < 1.000) samples and 235 grey-zone reactive discordant samples (microparticle enzyme immunoassay [MEIA] positive and EIA negative) were subjected to 'in-house' HBsAg neutralization assay ('in-house' NT). Using convenient sampling, 84 samples of adequate volume were compared with a commercial HBsAg neutralization assay.

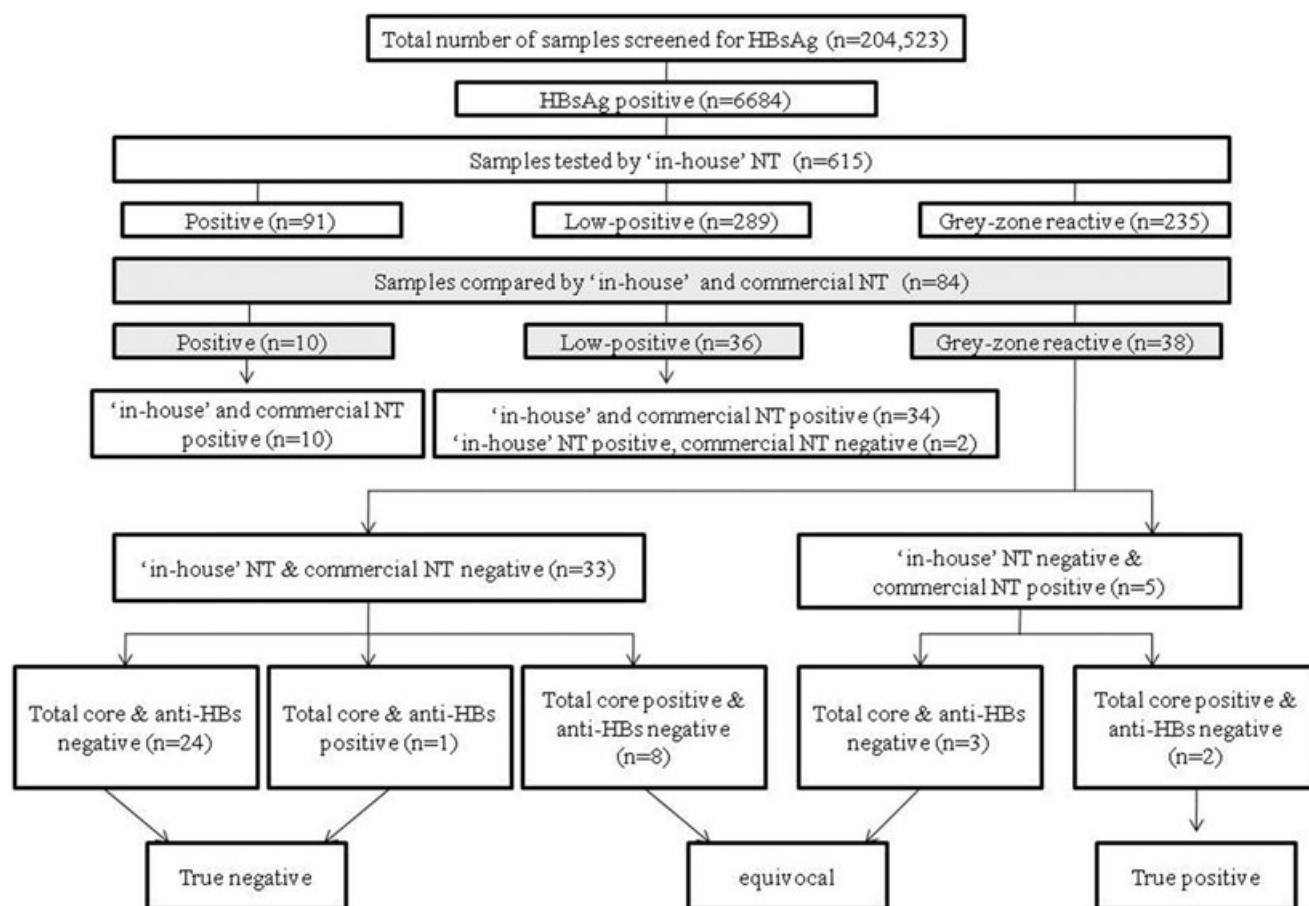


Figure 1 Flow chart of the study. Anti-HBs, anti-hepatitis B surface antibodies; HBsAg, hepatitis B surface antigen; NT, neutralization test.

All samples that were compared with the commercial neutralization assay were also tested for anti-HBc and anti-HBs to validate the HBsAg reactivity (reflex testing). The study flowchart is given in Figure 1.

HBsAg screening

Samples were centrifuged at 13 000 *g* for 10 min in a refrigerated centrifuge (4°C). HBsAg was screened using one of the three assays: ETI-MAK-4 (Diasorin, Saluggia, Italy), Hepanostika HBsAg Uni-Form II (BIOMERIEUX, Boxtel, the Netherlands) and HBsAg (V2) AxSYM system (Abbott, Weisbaden, Germany). Reactive samples were tested in duplicate in Diasorin and AxSYM to affirm the reactivity.

Preparation of neutralizing anti-serum

Blood samples of HBV vaccinees from the staff student health clinic (HIV, HCV and HBV negative) were screened for anti-HBs. Samples that showed an anti-HBs titer of 500 mIU/mL or more were pooled in a pool size of five. The pools were screened for HBsAg (ETI-MAK-4; Diasorin), anti-HBc (ETI-AB-COREK plus; Diasorin) and anti-HBs titer (Monolisa; Bio-Rad, Richmond, CA, USA). The pool that was negative for HBsAg and anti-HBc

with an anti-HBs titer of 1000 mIU/mL or more was used as a source of anti-HBs for the neutralization assay.

EIA-based HBsAg neutralization assay

Patient sera were equilibrated to room temperature and 250 μ L was transferred into three micro-centrifuge tubes (MCT). MCT-1 was used as a control to rule out the effects of incubation at 37°C on HBsAg. Negative control (250 μ L) was added to the second MCT (MCT-2) to factor for the change in OD values due to dilution effect. Equal amount (250 μ L) of anti-HBs pool was added to the third MCT (MCT-3). All tubes were vortexed, centrifuged briefly at 200 *g* and incubated at 37°C for an hour. After incubation, tubes were centrifuged at 4500 *g* for 10 min and loaded in duplicate into the respective wells and tested for HBsAg as per manufacturer instructions (ETI-MAK-4; Diasorin). Grading and interpretation criteria for 'in-house' neutralization assay are shown in Table 1.

Evaluation of 'in-house' HBsAg neutralization assay with commercial HBsAg confirmatory assay

The 'in-house' HBsAg confirmatory neutralization assay was compared with a commercial HBsAg confirmatory assay

Table 1 Grading and validation criteria for 'in-house' neutralization assay

MCT-1 OD	Criteria			Grading of HBsAg neutralization
	MCT-2 OD	MCT-3 OD	% of neutralization = $\frac{\text{MCT-2 OD} - \text{MCT-3 OD}}{\text{MCT-2 OD}} \times 100$	
1.000 to 3.000	> COV	< COV	> 90–100	Positive
> twice the COV to < 1.000	> COV	< COV	> 90–100	Low positive
> COV	> COV	> COV	< 50	Negative
< COV	< COV	< COV	Nil	Negative

COV, cut-off value; MCT-1 OD, sample control; MCT-2 OD, sample + negative control; MCT-3 OD, sample + anti-HBs; OD, optical density.

(AxSYM, HBsAg Confirmatory; Abbott). All patient samples were tested undiluted and also with a 1:500 dilution. The samples were transferred into four reaction vessels (RV) and treated with human anti-HBs (Reagent-A) and recalcified plasma (Reagent-B). The presence of non-neutralized HBsAg is determined by comparing the rate of formation of fluorescent product to a cut-off rate determined using the AxSYM HBsAg Confirmatory Index calibrator. If the rate of formation of fluorescent product in the non-neutralized sample (incubated with Reagent- B) is greater than or equal to the cut-off and the rate of the neutralized sample (incubated with Reagent-A) is reduced by at least 50%, the sample is considered positive.

Reflex tests

Anti-HBc estimation was performed using competitive EIA by ETI-AB-COREK (Diasorin). Anti-HBs estimation was done using Monolisa Anti-HBs 3.0 3 (Bio-Rad).

Results

Among the 204 523 samples screened for HBsAg using EIA- and MEIA-based assays, 6684 (3.3%) were positive. Of these, 'in-house' HBsAg NT assay was performed in 615 (9.2%) samples. Based on sample availability, 84 samples were compared with a commercial HBsAg confirmatory assay. The agreement between the two tests was excellent ($\kappa = 0.83$, $P < 0.001$) with 89.9% sensitivity and 94.3% specificity and the positive and negative predictive values were 95.7% and 86.8%, respectively.

Of the 615, 91 (15%) were high reactive (OD value > 1.000–3.000) and all these samples were neutralized in the 'in-house' HBsAg NT assay. In this reactivity range, 10 samples were compared with commercial HBsAg neutralization assay and all were neutralized with a neutralization rate of more than 90%. In reflex testing, all 10 samples were positive for anti-HBc and negative for anti-HBs.

Of the 289 (46%) samples that showed low reactivity in both EIA (OD < 1.000) and in MEIA during primary HBsAg screening, 283 (98%) showed 90–100% of neutralization in the 'in-house' HBsAg neutralization assay. Six samples that failed our 'in-house' neutralization assay were repeatedly HBsAg reactive in both the screening assays. Of the 289 samples, 36 available samples were compared with commercial neutralization assay. Thirty-four were positive in both the confirmatory assays and reflex tests. Two

samples that were positive in the 'in-house' neutralization assay but negative in commercial confirmatory were anti-HBs negative and anti-HBc positive.

Two hundred and thirty-five (38.2%) samples were reported as grey-zone positive/discordant reactive based on very low reactivity in MEIA and negativity in EIA. Of these, 38 samples with the reactivity of more than 2.76 to less than 26.03 S/N in the MEIA were subjected to the commercial HBsAg confirmatory assay. Thirty-three of these samples were 'in-house' and commercial neutralization assay negative. Of these, 24 were anti-HBc and anti-HBs negative, eight were anti-HBc positive and anti-HBs negative and one was both anti-HBc and anti-HBs positive (835 mIU/mL). The remaining five samples were 'in-house' NT negative and commercial confirmatory assay positive. All these five samples were anti-HBs negative and two were anti-HBc positive.

Discussion

Hepatitis B surface antigen is used for diagnosis of acute and chronic HBV infection. Every available commercial HBsAg detection kit emphasizes the need to confirm HBsAg reactivity by neutralization assays. To our knowledge, this is the first report that describes an 'in-house' confirmatory test for HBsAg which is important in screening and confirmation of HBsAg in clinical samples of low resource settings.

The concentration of HBsAg varies in the natural course of HBV infection.⁵ Based on the configuration of immunoassay employed, the detection limit can vary from 0.08–0.16 ng/mL.⁶ In our study, samples that had an OD value of more than 1.000 in EIA showed neutralization rate of more than 90% in the 'in-house' and commercial confirmatory assays. Hence, commercial HBsAg confirmatory assay and reflex tests do not have an additional advantage in such samples. This observation is supported by O'Brien who showed that HBsAg confirmation and reflex testing were not essential for highly reactive samples.⁷

Among 289 samples that were of low reactivity with an OD less than 1.000, 283 (98%) were 'in-house' NT positive. We speculate that negativity in the remaining six samples could be due to clonality of anti-HBs used for pool preparation or false positive. Due to inadequate sample volume, both commercial confirmatory assay and reflex tests were not performed in these samples. Two samples that were positive in the 'in-house' NT and negative in commercial confirmatory assay were anti-HBc positive and anti-HBs negative suggesting that they were likely to be true HBsAg positives. A

study from China has shown that low levels of HBsAg occur significantly in HBV-infected individuals.⁸ In our experience, 'in-house' NT is beneficial in confirming low-reactive samples but the role of commercial confirmatory and reflex tests are questionable.

In HBsAg screening, 235 (3.5%) samples were EIA negative and repeatedly MEIA positive. These samples were referred as 'grey-zone positive/discordant reactive'. Thirty-eight of the 235 grey-zone reactive samples (in MEIA) were included in the 'in-house' NT assay to maintain uniformity of testing. In this, a large proportion (87%) was commercial confirmatory assay negative. This suggests the occurrence of high false positivity associated with MEIA-based HBsAg screening assay, which has been shown in earlier studies.⁹ Biological false positives can also occur among HBsAg vaccinees and in individuals receiving granulocyte-colony stimulating factor (G-CSF).^{10,11} Only five of the 38 samples (13%) were positive in the commercial confirmatory assay. Of the five, two were anti-HBc positive and anti-HBs negative suggesting that they were true positives. The remaining three were anti-HBc and anti-HBs negative. This profile has been shown to occur in previous studies and interpreted as 'equivocal'.¹² In grey-zone positive/discordant reactive samples, reflex testing is essential to validate the HBsAg reactivities. Samples with this reactivity should be followed up and the results should be carefully interpreted in the light of symptomatology of the disease and other laboratory markers such as liver enzymes.

Thirty-three samples were negative in both confirmatory assays; of which 24 samples were anti-HBc and anti-HBs negative and interpreted as true negatives. Eight samples were anti-HBc positive and anti-HBs negative and interpreted as 'equivocal'. Biologically, these patients may have cleared the infection much earlier resulting in attrition of anti-HBs over time. However, occult HBV infection, serological core window and escape mutants cannot be ruled out and follow up is mandatory.¹³⁻¹⁵ The remaining one sample that was anti-HBc and anti-HBs positive (835 mIU/mL) was interpreted as a true HBsAg negative as described earlier.¹²

In conclusion, the role of 'in-house' NT in samples that have OD of more than 1.000 is optional and in samples of low positivity that have OD of less than 1.000 is extremely useful. Though the role of 'in-house' NT is limited in confirmation of grey-zone positive/discordant reactive samples, the need for a commercial confirmatory assay is not clear. However, follow up of such patients is mandatory. Low and high positive samples do not need reflex testing. In grey-zone positive/discordant reactive samples, reflex testing is helpful to resolve these reactivities. The 'in-house' NT is

a technically adaptable, cost-effective and a comprehensive approach to confirmatory HBsAg testing even in low resource settings, especially in samples showing low HBsAg positivity.

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