Clinical evaluation of a novel and highly sensitive immunoassay for anti-hepatitis B core antigen using a fully automated immunochemical analyzer

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Aim: Recently, the measurement of hepatitis B surface antigen and anti-hepatitis B core antigen (HBCAb) and/or anti-hepatitis B surface antigen has been recommended before various therapies to identify patients at risk of hepatitis B virus (HBV) reactivation. However, a recent study reported that HBV reactivation occurred in HBCAb-negative patients, indicating that it is challenging to identify patients with a history of HBV infection using conventional HBCAb reagent. We developed a highly sensitive HBCAb (HBCAb-HS) assay for reducing the risk of HBV reactivation.

Methods: The HBCAb-HS assay is an automated chemiluminescent enzyme immunoassay system, which is suitable for clinical use. The cut-off was set at 0.020 IU/mL from the distribution patterns of HBCAb-negative specimens, and we evaluated the performance of this assay compared with conventional reagents.

Results: This new assay showed a 27–81-fold greater sensitivity than conventional HBCAb reagents; the quantified measurement range was from 0.005 IU/mL to 1.500 IU/mL, and it showed excellent quantitative performance and correlated well with two conventional assays, using the HBCAb-positive specimens. Moreover, it showed 100% specificity for the 469 purchased HBCAb-negative specimens. Notably, this newly developed HBCab-HS assay showed positivity in the preserved specimens before HBV reactivation, for which conventional HBCAb reagents gave negative results, and the HBCab-HS assay could detect the lower HBCab levels even after intensive immunosuppressive therapies, including autologous hematopoietic stem cell transplantation.

Conclusions: The clinical efficacy of the newly developed, highly sensitive HBCab assay would enable the identification of patients at risk of HBV reactivation more accurately.

Key words: anti-hepatitis B core antigen, hepatitis B virus, hepatitis B virus reactivation, immunoassay

INTRODUCTION

It is estimated that there are approximately 240 million patients with chronic hepatitis B virus (HBV) and two billion people who have been infected with HBV.1,2 It has been established that patients with chronic HBV infection are at risk of HBV reactivation when they are exposed to immunosuppressive therapy or chemotherapy.3–5 However, recently, HBV reactivation has also been reported in patients with resolved HBV infection.3,6–12 Some reports indicate a correlation between HBV reactivation and host immunosuppression. Approximately
1.0–2.7% of patients treated with systemic chemotherapy, 12.2–23.8% of those treated with rituximab plus steroid, and 14.0–20.0% who underwent allogeneic hematopoietic stem cell transplantation from anti-hepatitis B core antigen (HBcAb)-positive and/or anti-hepatitis B surface antigen (HBsAb)-positive patients were shown to be at risk of HBV reactivation.\textsuperscript{3,7,9,10,12,13}

According to some experts, HBV reactivation in resolved HBV patients is defined as elevated HBV-DNA or presence of HBsAg with the use of immunosuppressive therapy or chemotherapy.\textsuperscript{14} Hepatitis B virus reactivation-related hepatitis tends to become severe and could require a change in therapy approach.\textsuperscript{15} Considering these risks, guidelines for HBV reactivation were published worldwide, and measurement of hepatitis B surface antigen (HBsAg), HBcAb, and/or HBsAb is recommended for establishing the diagnosis with respect to patients’ HBV infectious status or history of HBV infection, and for deciding the therapy strategy.\textsuperscript{14,16–19} Nevertheless, HBV reactivation was also reported in patients who tested negative for HBV serological makers.\textsuperscript{6,20,21} The reports suggest the need for more sensitive HBV markers for determining the history of HBV infection. To resolve this problem, we focused on HBcAb as evidence of HBV infection in those with previous exposure to HBV. In fact, HBcAb appears in the blood only in the case of HBV infection, whereas HBsAb becomes positive after HBV vaccination. We developed a new diagnostic marker using a highly sensitive HBcAb reagent (HBcAb-HS) and evaluated its clinical performance.

**METHODS**

**Samples and materials**

HEPATITIS B SURFACE antigen-negative, HBcAb-negative, and HBcAb-positive specimens were purchased from ProMedDx (Norton, MA, USA) and Trina Bioreactives (Naenikon, Switzerland). Original HBcAb results of these specimens were assessed using Architect Anti-HBc II (Abbott Laboratories, North Chicago, IL, USA). These samples were used to evaluate the basic performance of the HBcAb-HS assay in the specificity test, dilution test, and correlation study. The World Health Organization (WHO)’s first international standard for anti-hepatitis B core antigen, plasma, human (National Institute for Biological Standards and Control [NIBSC] code: 95/522) (WHO Standard for HBcAb) was purchased from NIBSC and diluted serially using the serum with HBsAg-negative, HBcAb-negative, and HBsAb-negative results. These serially diluted WHO standard samples were used as calibrators for determining the HBcAb levels in each specimen.

**Specimens of reactivated HBV infection**

Specimens from two myeloma patients (case 1 and case 2) with HBV reactivation were collected and preserved in Nagoya City University Hospital (Nagoya, Japan) and Aichi Medical University Hospital (Nagakute, Japan). The study was approved by the Ethics Committee of Nagoya City University Graduate School of Medical Sciences (approval reference: 00000683–4) and Aichi Medical University Hospital (approval reference: 2017-H148).

**Case 1**

This patient was a 60-year-old man diagnosed with symptomatic multiple myeloma (immunoglobulin A lambda [IgA-λ] type) who underwent autologous hematopoietic stem cell transplantation (ASCT) as an initial therapy. Before the ASCT, he was seronegative for HBsAg but seropositive for HBcAb and HBsAb, and HBV-DNA was not detected at baseline; therefore, HBV infection was determined as resolved, and he underwent regular HBV-DNA monitoring to prevent HBV-related hepatitis. He underwent systemic chemotherapy followed by ASCT. After ASCT, he experienced interstitial pneumonia and was treated with systemic steroids for approximately 8 weeks. Four months after ASCT, HBV reactivation occurred at HBV-DNA levels of 2.8 log copies/mL (peak HBV-DNA levels of 3.9 log copies/mL) without hepatitis. Entecavir, an anti-HBV nucleoside analogue, was given immediately, and HBV-DNA was decreased to below the limit of detection. Finally, he was treated with thalidomide and dexamethasone for relapsed myeloma and died due to suspected cardiac failure.

**Case 2**

The patient was a 57-year-old woman diagnosed with symptomatic multiple myeloma (IgA-κ type) who underwent ASCT as an initial therapy. Before ASCT, she was seronegative for HBsAg, HBcAb, and HBsAb. The multiple myeloma recurred approximately 5 months after the ASCT; therefore, she underwent local radiation therapy for bone lesions. Thereafter, for the refractory myeloma, she underwent various salvage systemic chemotherapies, including lenalidomide, bortezomib, carfilzomib, and dexamethasone. Post-transfusion screening blood test for infection revealed that HBV-DNA had increased up to 4.0 log copies/mL with no increase in the alanine aminotransferase levels at 21 months after the ASCT. Entecavir was given immediately, and HBV-DNA decreased to below the limit of detection. Finally, the patient died due to refractory myeloma 1 month after HBV reactivation was confirmed.
**Highly sensitive anti-hepatitis B core antigen assay**

Ferrite microparticles (Fujirebio, Tokyo, Japan) were coated with the recombinant hepatitis B core antigen (r-HBcAg) that was expressed from *Escherichia coli* (Fujirebio). Subsequently, this r-HBcAg-coated microparticle was blocked with bovine serum albumin (BSA). The particle solution was prepared as the suspension of this r-HBcAg-coated particle in particle diluent (2.0% BSA, 3.0% sucrose, 0.1% NaN₃, and 50 mM Tris-HCl; pH 7.2). For the alkaline phosphatase (ALP)-conjugate reagent, ALP-linked anti-human IgG monoclonal antibody was prepared using the hinge method and purified using chromatography with a HiLoad 16/600 Superdex 200 pg column (GE Healthcare, Wauwatosa, WI, USA). The conjugate solution was prepared by dilution of ALP-conjugate into the conjugate diluent (2.0% BSA, 0.1% casein sodium, 0.1% NaN₃, and 50 mM Tris–HCl; pH 7.5).

The HBcAb-HS assay was carried out using the Lumipulse Presto II (Fujirebio) automated chemiluminescent enzyme immunoassay (CLEIA) system. Fifty microliters of sample and 100 μL of the sample diluent (2.0% BSA, 3.0% sucrose, 0.1% NaN₃, and 50 mM Tris–HCl; pH 7.2) were mixed with 50 μL of the particle solution, and the mixture was incubated for 8 min at 37°C. After washing with Lumipulse Presto washing buffer (Fujirebio), the ferrite particle was subsequently incubated with the conjugate solution for 8 min at 37°C; thereafter, the relative luminescent intensity was measured by incubation for 4 min at 37°C with 200 μL substrate solution ([3-(2′-spiroadamanant)-4-methoxy-4-(3″-phosphoryloxy) phenyl-1,2-dioxetane disodium salt]; Fujirebio). In the HBcAb-HS assay, the calibration curve based on the WHO standard material was used to calculate the HBcAb levels in each specimen. In cases with the upper limit values >1.500 IU/mL, the specimen was diluted with sample diluent (2.0% BSA, 3.0% sucrose, 0.1% NaN₃, and 50 mM Tris–HCl; pH 7.2); thereafter, it was measured and calculated according to the dilution fold.

**Cut-off value**

We measured 476 HBsAg- and HBcAb-negative specimens to establish a cut-off value for the HBcAb-HS assay and analyzed the discordant samples with respect to the other HBV reagents results. Based on these analyses, seven samples that gave a negative result with the Architect Anti-HBcII reagent and a positive result with the HBcAb-HS assay were removed for determining the cut-off value. We analyzed the distribution pattern of the measurement values of the remaining 469 HBsAg-negative and HBcAb-negative specimens and tentatively set the cut-off value at 0.020 IU/mL that indicated the average + 6 standard deviations (SD) of the distribution.

**Inhibition test**

We established the inhibition test to verify the HBcAb-HS assay results. In brief, r-HBcAg was added to each specimen at a final concentration of 20 μg/mL. The inhibition rate (%) was calculated as follows: Inhibition rate (%) = ([HBcAb levels in the specimen] – [HBcAb levels in the r-HBcAg added specimen])/[HBcAb levels in the specimen] × 100.

**Decrease ratio before and after ASCT**

The decrease ratio (%) was calculated as follows: Decrease ratio (%) = ([HBcAb value after ASCT]/[HBcAb value before ASCT]) × 100.

**Other hepatitis B virus markers**

The measurements of HBsAg levels using Lumipulse HBsAg-HQ (Fujirebio) (cut-off value, 0.005 IU/mL) and HBsAb levels using Lumipulse HBsAb-N (cut-off value, 10 mIU/mL) were carried out using the Lumipulse G1200, a fully automated CLEIA system (Fujirebio), according to the manufacturer’s instructions. The measurement of HBcAb levels with the Lumipulse Presto HBcAb-III (cut-off value, 1.0 cut-off index) was carried out using the Lumipulse Presto II. The detection of HBsAg using Architect HBsAg QT (cut-off value, 0.05 IU/mL), HBsAb levels using Architect Ausab (cut-off value, 10.0 mIU/mL), and HBcAb levels using Architect Anti-HBcII (cut-off value, 1.0 signal to cut-off [S/CO]) were carried out according to the manufacturer’s instructions (Abbott Laboratories).

**RESULTS**

**General performance of HBcAb-HS assay**

The HBcAb-HS assay on the Lumipulse Presto II system was established by using the optimized reaction components, concentrations, and assay conditions. This assay is a two-step sandwich CLEIA using ALP-conjugated anti-human IgG as a tracer. The resulting reaction signals are derived within 20 min of the test; thus, it is suitable for clinical use. The calibration curve was prepared using serially diluted specimens of the WHO standard for HBcAb with HBsAg-negative, HBsAb-negative, and HBcAb-negative serum. It was shown that the reactivity of the HBcAb-HS assay depended on the HBcAb concentrations to 1.500 IU/mL. The analytical detection limit was 0.0013 IU/mL, and the point did not overlap with the concentration corresponding to 3 SD above the mean of the
chemiluminescence intensity of the zero calibrator (n = 20). Moreover, the limit of quantitation showed 0.005 IU/mL from the HBcAb concentration with 10.0% coefficients of variation in this assay. We tentatively settled from 0.005 IU/mL to 1.500 IU/mL as the quantitative measurement range. In addition, we set the tentative cut-off value of the HBcAb-HS assay to 0.020 IU/mL from the distribution of the purchased HBsAg-negative and HBcAb-negative specimens as described above (Fig. 1).

Comparison of three HBcAb assays with serially diluted specimens

Four HBcAb-positive specimens were serially diluted three times with HBsAg-negative, HBsAb-negative, and HBcAb-negative serum and were measured using the HBcAb-HS, Architect Anti-HBcII, and Lumipulse Presto HBcAb-III, respectively. Architect Anti-HBcII and Lumipulse Presto HBcAb-III were selected as typical conventional HBcAb reagents. From observed WHO Standard measurements, the cut-off value of Architect Anti-HBcII was approximately 0.7 IU/mL and approximately 0.6 IU/mL for the Lumipulse Presto HBcAb-III. The results were calculated and are shown in Table 1. The HBcAb-HS showed 27–81-fold higher sensitivity than Architect Anti-HBcII and 27-fold higher sensitivity than Lumipulse Presto HBcAb-III. The recovery rate of the HBcAb-HS assay ranged from 89.0% to 110.1%; thus, this assay showed a high quantity in the measurement range.

Specificity of the HBcAb-HS assay

The 476 HBsAg-negative specimens were measured using three reagents: HBcAb-HS assay, Architect Anti-HBcII, and Lumipulse Presto HBcAb-III (Table 2a,b). The distribution of the measurement values by HBcAb-HS assay is shown in Figure 1. The modal value was 0.001 IU/mL, and the SD was 0.002 IU/mL. Of the 476 specimens, 469 gave negative results for HBcAb with all HBcAb reagents (Table 2a,b). There were seven discordant specimens between the HBcAb-HS assay and Architect Anti-HBcII. Six of these seven specimens were also positive with Lumipulse Presto HBcAb-III, and one was positive with only HBcAb-HS assay. We analyzed these discordant samples by using three other assays, Lumipulse HBsAg-HQ that had the highest sensitivity to HBsAg, Lumipulse HBsAb-N, and the in-house inhibition test using the HBcAb-HS assay, as described above. The results of these seven discordant samples are presented in Table 2(c). Two of the seven specimens showed positive results with Lumipulse HBsAg-HQ, and all seven samples were HBsAb-negative with

Figure 1  Distribution of measurement values for purchased hepatitis B surface antigen-negative and hepatitis B core antibody (HBcAb)-negative specimens with conventional assays in the highly sensitive HBcAb (HBcAb-HS) assay. A total of 476 specimens were measured using the HBcAb-HS assay. The modal value of negative specimens was 0.001 IU/mL and the standard deviation (SD) was 0.002 IU/mL. Positive samples were subjected to inhibition tests. The cut-off value was set to 0.020 IU/mL. This value means the average + 6 SD of the distribution of the immunoreactivity. As the immunoreactivity in all negative specimens, except for seven samples judged to be positive by other HBV markers and inhibition test, was below this cut-off value, the specificity of the HBcAb-HS assay was determined as 100% at the 0.020 IU/mL cut-off.
Table 1  Sensitivity and quantity evaluation of three hepatitis B core antibody (HBcAb) assays with diluted HBcAb-positive specimens

| Sample no. | Dilution fold | HBCab-HS IU/mL Judge | Architect Anti-HBc II S/CO Judge | Lumipulse Presto HBcAb-III C.O.I. Judge |
|------------|---------------|----------------------|----------------------------------|----------------------------------------|
| 1          |               |                      |                                  |                                        |
| 1          |               | ≥1.500 +             | 12.10 +                          | 176.0 +                                |
| 10         |               | ≥1.500 +             | 10.40 +                          | 147.1 +                                |
| 100        |               | ≥1.500 +             | 10.10 +                          | 39.2 +                                 |
| 300        |               | ≥1.500 +             | 8.33 +                           | 13.0 +                                 |
| 900        |               | ≥1.500 +             | 5.37 +                           | 4.6 +                                  |
| 2700       |               | 1.367 +              | 2.07 +                           | 1.7 +                                  |
| 8100       |               | 0.432 +              | 0.75 −                           | 0.6 −                                  |
| 24 300     |               | 0.168 +              | 0.29 −                           | 0.3 −                                  |
| 72 900     |               | 0.051 +              | 0.15 −                           | 0.2 −                                  |
| 218 700    |               | 0.017 −              | 0.13 −                           | 0.1 −                                  |
| 656 100    |               | 0.012 −              | 0.10 −                           | 0.1 −                                  |
| 1 968 300  |               | 0.002 −              | 0.10 −                           | 0.1 −                                  |
| 2          |               |                      |                                  |                                        |
| 1          |               | ≥1.500 +             | 11.90 +                          | 254.7 +                                |
| 10         |               | ≥1.500 +             | 11.20 +                          | 241.2 +                                |
| 100        |               | ≥1.500 +             | 10.10 +                          | 148.2 +                                |
| 300        |               | ≥1.500 +             | 9.63 +                           | 68.0 +                                 |
| 900        |               | ≥1.500 +             | 8.86 +                           | 24.2 +                                 |
| 2700       |               | ≥1.500 +             | 5.70 +                           | 8.9 +                                  |
| 8100       |               | ≥1.500 +             | 2.52 +                           | 3.2 +                                  |
| 24 300     |               | 0.665 +              | 0.89 −                           | 1.1 +                                  |
| 72 900     |               | 0.199 +              | 0.33 −                           | 0.4 −                                  |
| 218 700    |               | 0.068 +              | 0.18 −                           | 0.2 −                                  |
| 656 100    |               | 0.021 −              | 0.12 −                           | 0.1 −                                  |
| 1 968 300  |               | 0.009 −              | 0.10 −                           | 0.1 −                                  |
| 3          |               |                      |                                  |                                        |
| 1          |               | ≥1.500 +             | 11.60 +                          | 247.3 +                                |
| 10         |               | ≥1.500 +             | 10.20 +                          | 212.9 +                                |
| 100        |               | ≥1.500 +             | 9.63 +                           | 64.1 +                                 |
| 300        |               | ≥1.500 +             | 8.80 +                           | 23.1 +                                 |
| 900        |               | ≥1.500 +             | 5.50 +                           | 8.4 +                                  |
| 2700       |               | ≥1.500 +             | 2.55 +                           | 3.0 +                                  |
| 8100       |               | 0.609 +              | 0.85 −                           | 1.0 +                                  |
| 24 300     |               | 0.205 +              | 0.32 −                           | 0.4 −                                  |
| 72 900     |               | 0.061 +              | 0.18 −                           | 0.2 −                                  |
| 218 700    |               | 0.020 +              | 0.13 −                           | 0.1 −                                  |
| 656 100    |               | 0.008 −              | 0.10 −                           | 0.1 −                                  |
| 1 968 300  |               | 0.003 −              | 0.10 −                           | 0.1 −                                  |
| 4          |               |                      |                                  |                                        |
| 1          |               | ≥1.500 +             | 11.20 +                          | 240.9 +                                |
| 10         |               | ≥1.500 +             | 10.50 +                          | 126.7 +                                |
| 100        |               | ≥1.500 +             | 7.13 +                           | 19.4 +                                 |
| 300        |               | ≥1.500 +             | 4.08 +                           | 6.2 +                                  |
| 900        |               | 0.944 +              | 1.60 +                           | 2.3 +                                  |
| 2700       |               | 0.307 +              | 0.60 −                           | 0.8 −                                  |
| 8100       |               | 0.113 +              | 0.26 −                           | 0.3 −                                  |
| 24 300     |               | 0.036 +              | 0.14 −                           | 0.2 −                                  |
| 72 900     |               | 0.012 −              | 0.11 −                           | 0.1 −                                  |
| 218 700    |               | 0.004 −              | 0.11 −                           | 0.1 −                                  |
| 656 100    |               | 0.002 −              | 0.09 −                           | 0.1 −                                  |
| 1 968 300  |               | 0.001 −              | 0.10 −                           | 0.1 −                                  |

Architect Anti-HBcII from Abbott Laboratories (North Chicago, IL, USA); Lumipulse Presto HBcAb-III from Fujirebio (Tokyo, Japan).
C.O.I., cut-off index; S/CO, signal to cut-off ratio.
Lumipulse HBsAb-N. In the in-house inhibition test, all of these specimens showed an inhibition ratio of $\geq 80.0\%$, indicating the specific binding activity against recombinant HBcAg. Based on these results, we concluded that these seven discordant samples were positive for HBcAb. Thus, Architect Anti-HBcII and Lumipulse Presto HBcAb-III showed false-negative results in seven of the 476 samples (98.5%) and in one of the 476 samples (99.8%) in this study. The newly developed HBcAb-HS assay showed 100% specificity in the 469 samples except for seven HBcAb-positive specimens in this study.

**Correlation among three HBcAb assays using HBcAb-positive specimens**

The 145 HBcAb-positive specimens were also measured by the three HBcAb detection assays, HBcAb-HS assay, Architect Anti-HBcII, and Lumipulse Presto HBcAb-III. In the HBcAb-HS assay, specimens with values $>1.500$ IU/mL were diluted with the sample diluent, and the measurement value was calculated as per the dilution. The concordance ratio among Architect Anti-HBcII, Lumipulse Presto HBcAb-III, and HBcAb-HS assay for the qualitative evaluation was 100%. In the correlation study, using each measurement value in the three kinds of HBcAb assays, the HBcAb-HS assay showed 0.47 with Architect Anti-HBcII and 0.85 with Lumipulse Presto HBcAb-III as a correlation coefficient (Fig. 2). Architect Anti-HBcII showed saturation at a high HBcAb value range ($>9$ S/CO). In the lower HBcAb value range, for example, 0.005–20.000 IU/mL, at which Architect Anti-HBcII did not show saturation, the concordance value was 0.86.

**Performance of HBcAb-HS assay using clinical samples**

The clinical utility of the HBcAb-HS assay was evaluated with two specimens collected before HBV reactivation

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**Table 2**  Concordance ratios among three HBcAb assays using HBsAg negative and HBcAb negative specimens with conventional assays and analysis of seven discordant specimens

(a)

| Architect Anti-HBc II | + | − | Total |
|-----------------------|---|---|------|
| HBcAb-HS              | + | 0 | 7    |
|                       | − | 0 | 469  |
|                       |   |   |      |
| Concordance ratio     |   |   | 98.5%|

(b)

| Lumipulse Presto HBcAb-III | + | − | Total |
|-----------------------------|---|---|------|
| HBcAb-HS                   | + | 6 | 1    |
|                            | − | 0 | 469  |
|                            |   |   |      |
| Concordance ratio          |   |   | 99.8%|

(c)

| Sample No. | Lumipulse Presto II HBsAg-HQ† | Lumipulse HBsAb-N‡ | HBcAb-HS | IU/mL | Judge | IU/mL | Judge | Inhibition ratio (%) |
|-------------|-------------------------------|--------------------|----------|-------|-------|-------|-------|----------------------|
| 1           | 0.001                         | 0.0                | 0.422    | +     | 97.9  |
| 2           | 0.001                         | 0.5                | 0.153    | +     | 80.7  |
| 3           | 0.009                         | 1.4                | 0.233    | +     | 100.0 |
| 4           | 0.001                         | 0.2                | 0.144    | +     | 100.0 |
| 5           | 0.029                         | 0.2                | 0.176    | +     | 100.0 |
| 6           | 0.001                         | 0.1                | 0.273    | +     | 98.7  |
| 7           | 0.001                         | 3.7                | 0.024    | +     | 90.8  |

†: HBsAg detection reagent. Cut off value: 0.005 IU/mL.
‡: HBsAb detection reagent. Cut off value: 10 mIU/mL.
from myeloma patients. Both patients received several chemotherapy regimens including ASCT for more than 1 year. Hepatitis B virus reactivation was confirmed by detection of HBV-DNA in the blood. Patient information has been summarized in Table 3. Specimens at two sampling points before HBV reactivation in each patient were measured using this assay. These assessments were retrospectively carried out before and after ASCT, as shown in Figure 3.

In case 1, the baseline HBsAg using Architect HBsAg QT (cut-off value, 0.05 IU/mL) and HBV-DNA using real-time polymerase chain reaction (cut-off value, 2.1 log copies/mL) were not detectable, but both HBsAb using Architect Anti-HBcII (cut-off value, 1.0 S/CO) and HBcAb using Architect Ausab (cut-off value, 10.0 mIU/mL) were detectable. The HBcAb-HS assay showed a positive result (0.310 IU/mL) even after ASCT, although Architect Anti-HBcII showed a negative result (Fig. 3a).

Table 3 Two cases of symptomatic multiple myeloma (MM) before and after hepatitis B virus (HBV) reactivation

| Case | 1 | 2 |
|------|---|---|
| **At baseline** |   |   |
| Age, years | 60 | 57 |
| Gender | Male | Female |
| Diagnosis | MM, IgA-λ | MM, IgA-κ |
| Anti-HBc, S/CO; with Architect Anti-HBcII | Positive | Negative |
| Anti-HBs, mIU/mL; with Architect Ausab | Positive | Negative |
| HBV-DNA (log copies/mL) | ND | NA |
| Number of MM treatment regimens | 2 | 7 |
| ASCT | Yes | Yes |
| **After HBV reactivation** |   |   |
| Timing at reactivation from enrollment, months | 7.9 | 29.2 |
| Timing at reactivation from ASCT, months | 3.7 | 20.5 |
| Peak HBV-DNA, log copies/mL | 3.9 | 4.6 |
| Reverse seroconversion of HBsAg | Yes | Yes |
| Hepatitis due to reactivation | No | No |
| Last HBV-DNA measurement, month | 29.5 | 30.7 |
| Outcome of myeloma | Unknown death | Death from MM |

Architect Anti-HBcII and Ausab from Abbott Laboratories (North Chicago, IL, USA). ASCT, autologous hematopoietic stem cell transplantation; HBsAg, hepatitis B surface antigen; IgA, immunoglobulin A; S/CO, signal to cut-off ratio.
In case 2, none of the HBV serological markers were detected (HBsAg, HBcAb, HBsAb using conventional assays, nor HBV-DNA), so the patient was judged to be uninfected with HBV and not at risk of HBV reactivation. Surprisingly, HBcAb was detectable (0.158 IU/mL) at the pre-ASCT using the HBcAb-HS assay, indicating that the patient had resolved HBV infection. Moreover, using this assay, HBcAb was also detectable (0.052 IU/mL) after the ASCT and after several salvage chemotherapies for refractory myeloma (Fig. 3b).

The presence of HBcAb in all specimens in this study was confirmed using the inhibition test; the results are summarized in Table 4. Specimens at each of the two points tested positive in case 1 and case 2; furthermore, the inhibition ratios were >80.0% (98.5–100%). These four specimens in case 1 and case 2 were determined as positive for HBcAb in the analyses. Focusing on the antibody titer, HBcAb and HBsAb showed similar decrease ratios at the two time points, before and after ASCT, in each case. In case 1, the decrease ratios for HBcAb and HBsAb were 20.1% and 24.4%, respectively. In case 2, these were 32.9% and 22.4%, respectively.

DISCUSSION

RECENTLY, HBV REACTIVATION has been reported not only in chronic HBV infected patients, but also...
in patients with resolved HBV infection following immunosuppressive therapy or systemic chemotherapy.\(^{3,6-12}\) For instance, in liver transplantation patients who received the liver of an HBcAb-positive donor,\(^{23}\) molecularly targeted drugs with immunosuppressive effects have been reported to be associated with some risk of HBV reactivation.\(^{24-26}\) Interestingly, as HBV reactivation has also been reported in both HBsAg-negative and HBcAb-negative patients,\(^{6,20,21}\) we addressed this issue by developing a new high-sensitivity HBcAb assay, the HBcAb-HS assay.

The new HBcAb-HS assay showed excellent performance in the measurement of HBcAb levels in this study. Using diluted versions of the WHO standards for HBcAb and HBcAb-positive specimens, this new HBcAb-HS assay showed 27–81-fold higher sensitivity than conventional HBcAb reagents. Notably, this newly developed HBcAb-HS assay showed positivity in the preserved specimens before HBV reactivation, for which conventional HBcAb reagents gave negative results. In fact, using the new assay, low titers of HBcAb were also detectable after several salvage chemotherapies and ASCT for relapsed/refractory myeloma. Hence, we expect that the highly quantitative HBcAb-HS assay could present new possibilities of clinical utility by using several clinical specimens even after intensive immunosuppressive therapies, including ASCT.

In the measurement of 145 HBcAb-positive specimens, all three HBcAb reagents showed 100% concordance with each other. Furthermore, the correlation study results indicated that the HBcAb-HS assay had a high correlation with the conventional HBcAb reagents. In clinical practice, the main function of this assay is to detect HBcAb in patients with resolved HBV infections who are scheduled to undergo immunosuppressive therapy or systematic chemotherapy. Here, we evaluated the effectiveness of the HBcAb-HS assay using specimens at two time points (before and after ASCT) from observed patients with HBV reactivation with immunosuppressive therapies. The HBcAb-HS assay detected HBcAb at both time points, indicating that the assay was able to achieve detection even after the patient had undergone several rounds of immunosuppressive therapy (Fig. 3). It is possible that the HBcAb-HS assay reflects the reduction of IgG in the blood, a consequence of immunosuppression. Considering these results, the HBcAb-HS assay might be able to provide information regarding the immune suppression of patients. Further investigation and evaluation is warranted to confirm whether the HBcAb-HS assay can be used to identify patients at risk of HBV reactivation based on the decrease in HBcAb. Moreover, it appears worthwhile to monitor HBsAg, HBsAb, HBcAb, and HBV-DNA values before and during HBV reactivation, and evaluate their potential association to patient prognosis.

In addition, in terms of observed HBcAb, the sensitivity was verified. Both sensitivity and specificity were assessed by evaluating the 476 HBsAg-negative and HBcAb-negative specimens with the HBcAb-HS assay, Architect Anti-HBcII, and Lumipulse Presto HBcAb-III (Table 2). Seven discordant specimens were verified to be positive using other HBV markers and the inhibition test. These results indicated that the HBcAb-HS assay had 100% specificity for this evaluation. The HBcAb-HS assay showed the potential to be used as an alternative to the conventional HBcAb reagent. Moreover, a specificity study with a larger sample of clinical specimens should be undertaken to validate the cut-off value. The specificity examination, using both HBsAg-negative and HBcAb-negative specimens, with the current conventional reagents, showed an estimated 1.5% of resolved HBV patients (7 of 476) were misdiagnosed as being uninfected. In addition, this result indicates that the global prevalence of HBV infection might be higher than reported; therefore, further research and clinical evaluations pertaining to this subject are warranted. Simultaneously, we also developed an inhibition test by the addition of recombinant HBcAg to determine

Table 4 Characters of hepatitis B virus (HBV) markers before and after autologous hematopoietic stem cell transplantation (ASCT) in two cases of symptomatic multiple myeloma

| Case | ASCT | S/CO | Judge | HBcAb-HS | Judge | Inhibition ratio, % | Lumipulse HBsAb-N | Judge |
|------|------|------|-------|----------|-------|---------------------|--------------------|-------|
| 1    | Before | 2.31 | +     | 1.542    | +     | 100.0               | 59.1               | +     |
|      | After  | 0.57 | –     | 0.310    | +     | 99.3                | 14.4               | +     |
| 2    | Before | 0.29 | –     | 0.158    | +     | 98.5                | 9.8                | –     |
|      | After  | 0.13 | –     | 0.052    | +     | 100.0               | 2.2                | –     |

Architect Anti-HBcII from Abbott Laboratories (North Chicago, IL, USA); Lumipulse HBsAb-N from Fujirebio (Tokyo, Japan). HBcAb-HS, highly sensitive hepatitis B core antibody assay; S/CO, signal to cut-off ratio.
the specificity of the immunoreaction. We also tried to detect HBCAb using western blotting; however, it had low sensitivity and was unable to detect HBCAb at low concentrations.

The Lumipulse HBsAg-HQ is a commercially available reagent with the highest sensitivity for HBsAg detection (Table 2c). A highly sensitive HBsAg detection assay might be of benefit in terms of prevention of HBV reactivation. Therefore, an assay combining a higher HBsAg detection assay and higher HBCAb assay might be very useful for identifying patients at risk of HBV reactivation.

In conclusion, we reported the high sensitivity and specificity of the newly developed HBCAb-BS assay in this study. This new assay showed considerably higher sensitivity for detecting even minute levels of HBCAb, compared with conventional assays in various studies, including clinical specimens from HBV-reactivated patients. Hence, the clinical efficacy of the newly developed highly sensitive HBCAb assay would enable the identification of patients at risk of HBV reactivation more accurately.

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