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Protocols

# A novel immunoassay for PreS1 and/or core-related antigens for detection of HBsAg variants

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ABSTRACT

A novel immunoassay that detects simultaneously hepatitis B virus (HBV) PreS1 and/or core-related antigens was developed and evaluated for its potential for detecting hepatitis B surface antigen (HBsAg) variants. The detection limits of the assay was $10^{2.9\pm0.5}$ copies/mL (mean ± SD) for HBsAg-positive sera with different genotypes, and $10^{3.5\pm1.2}$ copies/mL for HBsAg variants sera. The specificity of the assay was 99.9% (95% CI: 99.7–99.9%, 4551 healthy individuals). The sensitivities were 93.9% (95% CI: 92.8–94.9%), 59.3% (95% CI: 38.7–77.6%) and 80% (95% CI: 44.4–97.5%) in three independent groups which include: 2065 hepatitis patients, 27 patients with occult hepatitis B and 10 HBsAg variants, respectively. In addition, a novel premature stop code mutation at position 112 of HBsAg was observed in two patients with chronic hepatitis B with different genotypes.

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## 1. Introduction

The surface antigen of HBV (HBsAg) composed of 226 amino acids is the most common diagnostic marker for HBV infections. HBsAg also serves as the protective antigen in the hepatitis B vaccine that is used globally for preventing HBV infections (Kao and Chen, 2002; Zuckerman, 1990). The highly conserved “a” epitope (amino acids (aa) 124–147) of HBsAg (Brown et al., 1984; Manivel et al., 1992), located within the major hydrophilic region (MHR, aa 100–160) of the S protein (Takeshima et al., 1985), is the major immune target for antibodies that are used either for immunoprophylaxis (Neurath, 1975) or in immunoassays for the detection of HBsAg. Many forms of HBsAg mutants have been reported. This is likely due to the immune response against HBV, global hepatitis B vaccination programs (Carman et al., 1990; Cooreman et al., 2001; Harrison et al., 1991), increasing post-exposure prophylaxis with hepatitis B immune globulin (HBIG) (Carman et al., 1999; Protzer-Knolle et al., 1998), and the increasing use of antiviral therapy with nucleoside or nucleotide analogs (Pollicino et al., 2009). Some of the mutation sites which occur in the MHR will lead to significant antigenic change of the “a” epitope, which may further result in a false negative result in common HBsAg assays (Coleman et al., 1999; Louisirirotchanakul et al., 2004; Weber, 2005).

Currently, the diagnosis of HBsAg-negative infection depends mainly on nucleic acid testing (NAT) that requires cumbersome procedures and expensive equipment. The development of novel immunoassays that can detect the HBsAg antigenic variants are needed in order to improve the diagnosis of these patients, especially for developing countries which cannot afford the high cost NAT testing.

The large HBsAg protein includes the PreS1 region, the PreS2 region, and the S gene. The PreS1 protein appears to be an essential components of the mature virion envelope and can also be found in the filament particles (Neurath et al., 1985). The presence of the PreS1 proteins in serum or liver has been considered a marker of HBV replication (Le Guillou et al., 2000; Petit et al., 1990; Theilmann et al., 1986). HBeAg is another infection marker of HBV in addition to HBsAg, and its level in serum is closely related to the titer of HBV. The core protein (HBcAg) is a part of viral particle,

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and since HBeAg and HBcAg share a 149-amino-acid region, they are called collectively hepatitis B core-related antigens (HBcrAg) (Kimura et al., 2002; Rokuhara et al., 2003). Hence, the detection of the PreS1 protein and/or HBcrAg in serum may serve as an indicator of HBV infection even in presence of HBsAg variants (Yuan et al., 2007). In this study, a novel double-sandwich immunoassay (Combo ELISA) was developed to detect simultaneously PreS1 and HBcrAg, and are described its potential to detect HBsAg variants was also included.

## 2. Materials and methods

### 2.1. Monoclonal antibodies

Monoclonal antibodies (MAbs) against PreS1 or HBcAg were prepared using hybridoma technology. Two anti-PreS1 MAbs, 7BH11 and 4D11, recognized two epitopes located on aa 21–47 of PreS1. Anti-HBc MAbs 20B11 and Cz recognized epitopes located on aa 62–75 and aa 90–149 of HBcAg, respectively. HBeAg is also well recognized by MAbs 20B11 and Cz. MAbs 4D11 and Cz were conjugated with HRP by the $NaIO_4$ oxidation method followed by gel filtration chromatography on a Superdex 200HR column. The purified mAb-HRP sample was mixed with an equal volume of glycerine and stored at −20 °C.

### 2.2. Immunoassays for PreS1 and/or HBcrAg

A lysis solution used for releasing the PreS1 protein and core antigens from HBV virus in the serum contained 2% HP-β-CD (hydroxypropyl-beta-cyclodextrin), 0.5% Triton X114, 0.5% Tween 20, 2 mM TECP·HCl (tris(2-carboxyethyl)-phosphine·HCl), and 1% β-2me (2-hydroxy-1-ethanethiol), and all these compounds were dissolved in 20 mM HEPES 7.0 buffer.

The Combo ELISA for PreS1Ag and/or HBcrAg involved the following steps: 96 microtiter well plates (Yixinmei; Xiamen, China) were coated with 100 μL of a mixture of anti-PreS1 MAb 7BH11 and anti-HBc MAb 20B11 and these solutions were left overnight. Thereafter, the wells were washed twice with phosphate-buffered saline (PBS, pH 7.4) followed by incubation with a blocking reagent (10% sucrose, 1% casein-Na, and 2% BSA in PBS, pH 7.4) at 37 °C for 2 h. After removing the blocking solution, the wells were vacuum dried and stored at 4 °C. A 50-μL lysis solution was added to each well followed by 50 μL of serum sample and incubated for 60 min at 37 °C. After incubation, the plates were washed five times with a wash buffer (0.05% Tween 20 in PBS, pH 7.4). Then a 100 μL aliquot of HRP-conjugated mixture composed of HRP-4D11 and HRP-Cz was added to each well and incubated for 30 min at 37 °C. After five times washing with the washing buffer, 100 μL of TMB substrate solution was added and the mixture were incubated for 15 min at 37 °C. The absorbance was then measured at 450 nm and 620 nm using a microplate reader (Tecan, Switzerland).

Immunoassays for PreS1 (PreS1-ELISA) and HBcrAg (HBcrAg-ELISA) were established similarly. In the PreS1-ELISA, MAb 7BH11 was used as a capture antibody and MAbs 4D11 was used as a second antibody. In the HBcrAg-ELISA, MAb 20B11 and Cz were used as the first and second antibodies, respectively. The cutoff value for each assay was set to the OD value of the negative control well plus 0.12.

### 2.3. PreS1 antigen, core-related antigen, and hepatitis B virus

Recombinant PreS1 protein (rPreS1, six-time-repeats of the aa 21–47 fragment) and HBcrAg (rHBcrAg, aa 1–149) were expressed and purified in *Escherichia coli*. Purified hepatitis B virus was supplied by the Wuhan Institute of Biologic Products (Wuhan, China).

### 2.4. Serum samples

After approval by the local bioethics committees, serum samples from 2363 patients with chronic hepatitis were collected. Serum samples of disease-free population were collected from 4968 workers employed in the food industry. Informed consent was obtained from all subjects. Each sample was divided into two aliquots prior to testing. One aliquot was used to detect the serological markers. Another aliquot was used to detect HBV DNA. Samples from the disease-free population were also submitted to HBV DNA PCR assay, in case at least one positive result identified in Combo ELISA, HBsAg, or HBeAg assay. Five plasma samples carrying S gene MHR mutants were collected from blood donors in Xiamen, China.

### 2.5. HBV markers of infection

HBsAg was first detected using a commercial ELISA kit (Wantai, Beijing, China). The negative clinical samples were retested using another commercial HBsAg kit (V3, Abbott Murex, UK). Anti-HBs, HBeAg, anti-HBe, and anti-HBc were detected using commercial ELISA kits (Wantai, Beijing, China). All assays were performed according to the manufacturer's instructions.

### 2.6. HBV DNA detection and sequencing

HBV DNA was detected initially using a commercial realtime-PCR (q-PCR) (Da'an, Guangzhou, China) with a threshold of 500 copies/mL. Q-PCR negative samples were further submitted to a four sets of nested PCR (n-PCR). The PCR products were sequenced for MHR (aa 110–165) directly by an ABI 3100 DNA automated sequencer (Applied Biosystem, California, USA) by a commercial service (Invitrogen, Shanghai, China). The primers and the protocols for nested PCR and DNA sequencing were described previously (Yuan et al., 2009).

### 2.7. Determination of genotype, serotype, and S gene MHR mutation

HBV genotype was identified by phylogenetic analysis using the neighbor-joining method (MEGA, v3.1). The analysis included HBV sequences with different genotypes obtained from GenBank. The reliability of the phylogenetic tree was tested using the bootstrap test with 1000 replicates. S gene MHR mutations were determined by comparison with HBV reference sequences from the relevant genotypes. HBV serotype was deduced from the presence of certain amino acids at positions 122, 127, 134, 159, and 160 (Norder et al., 2004).

## 3. Results

### 3.1. Evaluation and standardization of the Combo ELISA

The standard curves of SCO versus viral loads for the Combo ELISA, PreS1-ELISA, and HBcrAg-ELISA were established using purified hepatitis B virus as antigen targets (Fig. 1A). Samples of every dilution were tested in 3 microtiter wells and the mean SCOs were then calculated. The lowest threshold of detection was $1.0 \times 10^3$ copies/mL for Combo ELISA, $2.0 \times 10^3$ copies/mL for PreS1 ELISA, and $3.2 \times 10^4$ copies/mL for HBcrAg ELISA. The lysis solution can thus enhance dramatically the signals of both PreS1 and HBcrAg.

The standard Combo ELISA curves were established using recombinant PreS1 antigen (rPreS1) or HBcrAg (rHBcrAg) as antigen targets (Fig. 1B). The sensitivities for PreS1 and HBcrAg detection were 0.039 ng/mL and 0.020 ng/mL, respectively.

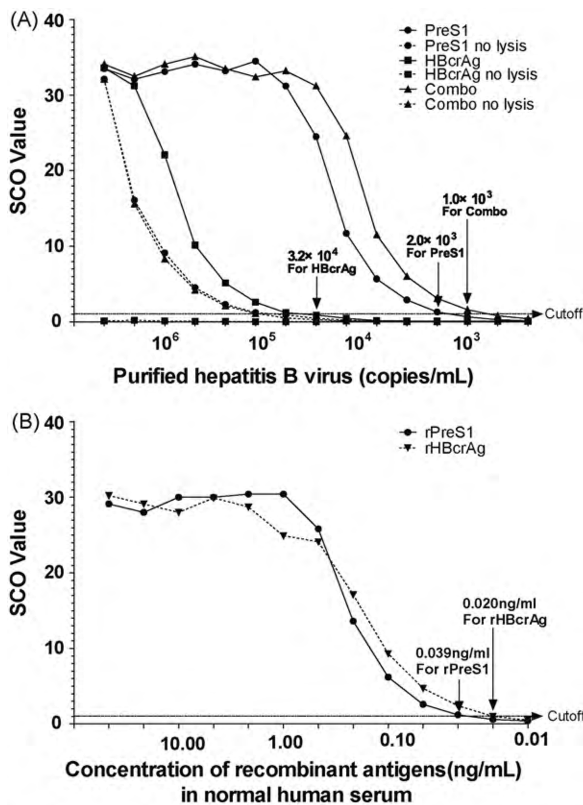


**Fig. 1.** (A) The calibration curves of the Combo ELISA, PreS1 ELISA, and HBcrAg ELISA obtained with purified HBV at decreasing dilutions. Stable lines show the results of assays with lysis solution and dashed lines show the results of assays without lysis solution. (B) The calibration curves of the Combo-ELISA obtained with recombinant PreS1 antigen and HBcrAg (HBc149).

### 3.2. Detection limits of various HBV antigen markers in hepatitis B sera

The detection limits of the Combo ELISA, PreS1 ELISA, and HBcrAg ELISA, HBeAg ELISA and HBsAg ELISA for hepatitis B sera were compared by a limiting-dilution assay. Five HBsAg-positive hepatitis sera (including genotype B2, C1, D1 and Ae) and five HBsAg mutants sera (mutated in MHR of S gene) were serially diluted and measured for HBV antigen markers. All HBV antigen values were evaluated by a signal/cutoff (SCO) index: an SCO value of over 1.0 indicated a positive response. The results are summarized in Tables 1 and 2. In all serum samples infected by wild type "S" region virus of genotype A, B, C and D (XNP1–XNP5), the Combo assay demonstrated a higher sensitivity than PreS1, HBcrAg or HBeAg assay, but a lower sensitivity than HBsAg assay. For 5 samples, the calculated detection limit for Combo assay was ranged from $4.4 \times 10^2$ copies/mL (XNP2) to $3.0 \times 10^3$ copies/mL (XNP5) (mean ± SD: $10^{2.9\pm0.5}$ copies/mL). This assay was able to detect HBcrAg even in anti-HBe and anti-HBc positive sera such as XNP1, which yielded a poor result with current HBeAg assay. The Combo assay was then also able to detect effectively the S variants contained serum samples which might yield negative or very low positive result in common HBsAg assay (Table 2, XMP1–XMP5). The calculated detection limit for Combo ELISA in detecting these S variants was ranged from $3.6 \times 10^2$ copies/mL (XMP2) to $1.2 \times 10^5$ copies/mL (XMP3) (mean ± SD: $10^{3.5\pm1.2}$ copies/mL). Mostly, the sensitivity of the PreS1 assay was about 10- to 100-fold higher than HBcrAg assay except for some samples such as XMP3 and XMP5, where the HBcrAg assay was superior.

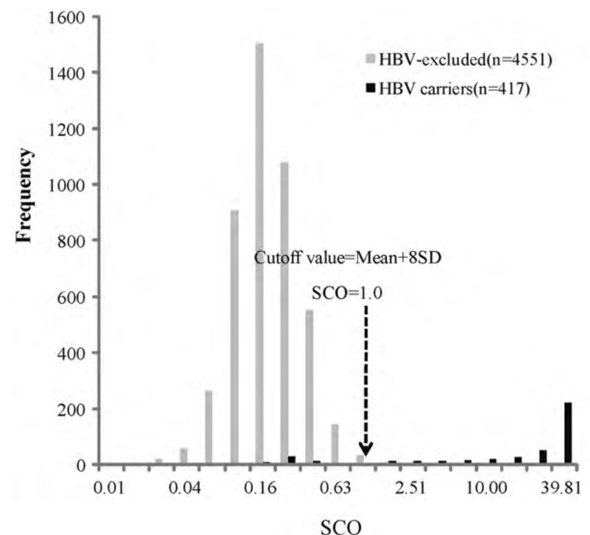


**Fig. 2.** The distribution of reactivity by the Combo-ELISA in HBV carriers for a population without for live HBV infection (HBV-excluded). The SCO values (*X*-axis) are shown as log-rank.

### 3.3. Specificity of Combo ELISA in the healthy population

Serum samples from 4968 healthy individual were tested for HBV infection markers. Samples positive for HBsAg, HBeAg, and/or by the Combo ELISA was tested further for HBV DNA by PCR. A total of 417 individuals were positive by the HBsAg and/or HBV DNA assays. Among the 4551 negative individuals, seven were Combo ELISA positive (Fig. 2). In these seven cases, neither HBsAg nor HBV DNA were detected, and five were also negative for anti-HBc. Hence, these cases were regarded as false positives for Combo ELISA and the specificity was calculated as 99.9% (95% CI: 99.7–99.9%). Among 417 HBV carriers, five were positive for HBV DNA but negative for HBsAg and these cases were then determined to have occult HBV infections. Three were found to be HBsAg variants carriers (Table 3). All the five occult infection cases were Combo ELISA positive.

### 3.4. Detection of HBsAg variants in patients with chronic hepatitis

Among 2363 chronic hepatitis patients, 271 patients (11.5%) were negative in both HBsAg and HBV DNA and were designated as Non-B hepatitis. All Non-B patients were negative by the Combo ELISA and hence the specificity of the assay (100%, 95% CI: 98.7–100%) was similar to that of Fig. 2 cited ($p = 0.582$). There were 2092 hepatitis B patients, 2065 (98.7%) were overt infections with positive HBsAg, and the other 27 (1.3%, 95% CI: 0.9–1.9%) were HBsAg negative. The MHR region of S gene from the 27 patients were sequenced and compared with the reference HBV sequences of relevant genotypes (Table 3). 10 patients (37.0%) with mutations in MHR region were identified and were considered as HBsAg variants.

The sensitivity of the Combo ELISA was 93.9% (1939/2065, 95% CI: 92.8–94.9%) in 2065 HBsAg-positive hepatitis patients, 59.3% (16/27, 95% CI: 38.7–77.6%) in 27 occult HBV patients and 80.0% (8/10,95% CI: 44.4–97.5%) in 10 HBsAg variants.

## 4. Discussion

HBsAg is used as a marker for HBV infection. However, individuals infected with HBsAg mutant(s) are negative in common HBsAg assay. Current methods for detecting such mutants are expensive and require trained staff and this prevents its application in devel-

**Table 1**
Dilutions of HBsAg-positive sera with different genotypes.

| ID | Genotype/serotype/S mut (anti-HBs/anti-HBe/anti-HBc) HBV DNA (copies/mL) | Dilution[a] | Signal/cutoff index | | | | |
|---|---|---|---|---|---|---|---|
| | | | Combo | Pre S1 | HBcrAg | HBeAg | HBsAg |
| XNP1 | Ba/adw2/wild | 0 | 27.4 | 33.2 | 28.1 | – | 32.5 |
| | (–/+/+) | 1:200 | 22.1 | 20.2 | 1.4 | Untested | 33.2 |
| | $5.4 \times 10^6$ | 1:2000 | 3.4 | 2.2 | – | Untested | 32.1 |
| | | 1:4000 | 1.7 | 1.2 | Untested | Untested | 33.4 |
| | | 1:8000 | – | – | Untested | Untested | 12.1 |
| | Calculated detection limit (copies/mL)[b] | | $8.0 \times 10^2$ | $1.1 \times 10^3$ | $2.0 \times 10^4$ | | |
| XNP2 | C1/adrq+/wild | 0 | 33.4 | 34.5 | 35.1 | 22.6 | 34.5 |
| | (–/–/+) | 1:200 | 15.9 | 11.2 | 1.2 | – | 33.1 |
| | $1.6 \times 10^6$ | 1:2000 | 1.8 | 1.1 | – | Untested | 32.4 |
| | | 1:4000 | – | – | Untested | Untested | 31.5 |
| | Calculated detection limit (copies/mL) | | $4.4 \times 10^2$ | $7.3 \times 10^2$ | $6.7 \times 10^3$ | | |
| XNP3 | D1/ayw1/wild | 0 | 35.2 | 34.1 | 33.5 | 33.1 | 32.5 |
| | (–/–/+) | 1:4000 | 34.1 | 31.5 | 25.6 | – | 35.4 |
| | $3.6 \times 10^9$ | 1:40000 | 33.5 | 32.6 | 2.4 | – | 35.1 |
| | | 1:800000 | 2.5 | 1.4 | – | Untested | 33.5 |
| | | 1:1600000 | 1.1 | – | Untested | Untested | 32.1 |
| | | 1:32000000 | – | Untested | Untested | Untested | 15.6 |
| | Calculated detection limit (copies/mL) | | $2.0 \times 10^3$ | $3.2 \times 10^3$ | $3.8 \times 10^4$ | | |
| XNP4 | C1/ayr/wild | 0 | 21.1 | 19.7 | – | – | 33.4 |
| | (–/+/+) | 1:10 | 2.3 | 1.2 | Untested | Untested | 15.1 |
| | $5.0 \times 10^3$ | 1:20 | – | – | Untested | Untested | 8.7 |
| | Calculated detection limit (copies/mL) | | $2.1 \times 10^2$ | $4.2 \times 10^2$ | – | | |
| XNP5 | Ae/adw2/wild | 0 | 11.2 | 7.6 | 1.3 | – | 33.1 |
| | (–/+/+) | 1:10 | 1.4 | – | – | Untested | 32.5 |
| | $4.2 \times 10^4$ | 1:20 | – | Untested | Untested | Untested | 34.1 |
| | Calculated detection limit (copies/mL) | | $3.0 \times 10^3$ | $5.5 \times 10^3$ | $3.2 \times 10^4$ | | |

[a] All serum samples diluted in diluted in normal human serum.
[b] Calculated detection limit (copies/mL) = "Original DNA load"/("Detectable limit dilution fold" × "limit SCO").

oping countries. Recently, it was noted that the serological pattern of anti-HBc positives alone (without HBsAg and anti-HBs) could be a possible marker of HBsAg-negative HBV infection (Grob et al., 2000; Vitale et al., 2008). However, using "anti-HBc alone" as a marker has low specificity since HBV DNA sequences were found only in about 5–10% of these samples (Candotti et al., 2008). Furthermore, such an approach is not applicable for high HBV prevalence countries. Hence, the development of simple methods for detecting mutant HBV strains is desirable.

Considering the high level of PreS1 antigens and HBcrAg in HBV infected individual and the level has no relation to HBsAg, it is expected that PreS1 and HBcrAg may help detect HBsAg-negative variants. Several assays have been developed for the measurement of serum PreS1 antigen (Le Guillou et al., 2000). However, in these

**Table 2**
Dilution test of HBsAg-negative variants sera.

| ID | Genotype/serotype/S mut (anti-HBs/anti-HBe/anti-HBc) HBV DNA (copies/mL) | Dilution[a] | Signal/cutoff index | | | | |
|---|---|---|---|---|---|---|---|
| | | | Combo | Pre S1 | HBcrAg | HBeAg | HBsAg |
| XMP1 | C1/adrq+/G145R | 0 | 12.1 | 11.2 | 2.2 | – | – |
| | (+/–/+) | 1:10 | 1.3 | 1.0 | – | Untested | Untested |
| | $1.3 \times 10^5$ | 1:20 | – | – | Untested | Untested | Untested |
| | Calculated detection limit (copies/mL)[b] | | $1.0 \times 10^4$ | $1.3 \times 10^4$ | $5.9 \times 10^4$ | | |
| XMP2 | C1/adrq+/S136P,G145R,C137R | 0 | 1.4 | 1.3 | – | – | – |
| | (+/–/–) | 1:2 | – | – | Untested | Untested | Untested |
| | $5.0 \times 10^2$ | | | | | | |
| | Calculated detection limit (copies/mL) | | $3.6 \times 10^2$ | $3.8 \times 10^2$ | – | | |
| XMP3 | B2/adw2/T131N,M133T,T140I | 0 | 7.8 | 2.1 | 8.2 | 3.3 | 1.9 |
| | (+/–/+) | 1:2 | 4.1 | – | 4.5 | 1.6 | – |
| | $9.7 \times 10^5$ | 1:4 | 1.9 | Untested | 2.1 | – | – |
| | | 1:8 | – | Untested | – | Untested | Untested |
| | Calculated detection limit (copies/mL) | | $1.2 \times 10^5$ | $4.6 \times 10^5$ | $1.1 \times 10^5$ | | |
| XMP4 | Ba/adw2/T126S | 0 | 3.5 | 2.8 | – | – | – |
| | (–/+/+) | 1:2 | 1.6 | 1.2 | Untested | Untested | Untested |
| | $5.0 \times 10^2$ | 1:4 | – | – | Untested | Untested | Untested |
| | Calculated detection limit (copies/mL) | | $1.6 \times 10^2$ | $2.1 \times 10^2$ | – | | |
| XMP5 | B2/adw2/T140I | 0 | 12.8 | – | 11.7 | 10.3 | – |
| | (–/–/+) | 1:10 | 1.3 | Untested | 1.4 | 1.0 | Untested |
| | $8.0 \times 10^4$ | 1:20 | – | Untested | – | – | Untested |
| | Calculated detection limit (copies/mL) | | $6.1 \times 10^3$ | – | $5.7 \times 10^3$ | | |

[a] All serum samples diluted in diluted in normal human serum.
[b] Calculated detection limit (copies/mL) = "Original DNA load"/("Detectable limit dilution fold" × "limit SCO").

**Table 3**
Characteristics of occult HBV infection cases determined in the study.

| Case code | Clinical[a] | City | Sex/age | HBsAg/anti-HBs/ HBeAg/anti-HBe/ anti-HBc | Viral load (copies/mL) | SCO of Combo | Genotype/serotype | Mutation in MHR | Refer sequence |
|---|---|---|---|---|---|---|---|---|---|
| YA114 | CHB | Beijing | F/20 | −/−/+/−/+ | $2.3 \times 10^7$ | 29.2 | C1/adrq+ | P111Q,G112stop[b] | AF286594 |
| YA665 | CHB | Beijing | M/37 | −/−/+/−/+ | $8.8 \times 10^4$ | 31.5 | C1/adrq+ | S113Q,S117N,T118R | AF286594 |
| YA783 | CHB | Beijing | M/43 | −/+/−/−/+ | <500 | 6.5 | Ba/adw2 | G145R | AB073826 |
| YA827 | CHB | Beijing | F/10 | −/+/−/−/+ | <500 | 3.4 | C1/adrq+ | S117G,A159T | AF286594 |
| YA387 | CHB | Beijing | F/12 | −/+/−/−/+ | <500 | – | C1/adrq+ | C124Y | AF286594 |
| YA53 | CHB | Beijing | M/31 | −/+/+/−/+ | <500 | 1.3 | Ba/adw2 | Wildtype | AB073826 |
| YA716 | CHB | Beijing | M/12 | −/−/−/+/+ | <500 | 1.5 | C1/adrq+ | Wildtype | AF286594 |
| YA577 | CHB | Beijing | F/32 | −/−/−/−/+ | <500 | 1.4 | C1/adrq+ | Wildtype | AF286594 |
| YA624 | CHB | Beijing | F/27 | −/+/−/+/+ | <500 | 2.4 | C1/adrq+ | Wildtype | AF286594 |
| YA646 | CHB | Beijing | M/17 | −/+/−/+/+ | <500 | – | C1/adrq+ | Wildtype | AF286594 |
| YA654 | CHB | Beijing | M/15 | −/−/−/+/+ | $8.0 \times 10^3$ | – | C1/adrq+ | Wildtype | AF286594 |
| YA693 | CHB | Beijing | M/51 | −/−/−/+/+ | <500 | – | C1/adrq+ | Wildtype | AF286594 |
| YA754 | CHB | Beijing | M/30 | −/−/−/+/+ | <500 | – | C1/adrq+ | Wildtype | AF286594 |
| YA822 | CHB | Beijing | F/23 | −/+/−/−/+ | <500 | – | C1/adrq+ | Wildtype | AF286594 |
| YA886 | CHB | Beijing | M/41 | −/−/−/+/+ | $3.0 \times 10^3$ | – | C1/adrq+ | Wildtype | AF286594 |
| WH22 | CHB | Wuhan | M/37 | −/−/−/−/+ | $1.0 \times 10^3$ | 23.5 | Ba/adw2 | C138R | AB073826 |
| XJK22 | CHB | Xi'an | M/41 | −/+/−/−/+ | $6.8 \times 10^7$ | 30.1 | Ba/adw2 | P111Q,G112stop# | AB073826 |
| XJU9 | CHB | Xi'an | M/28 | −/−/−/−/+ | $1.0 \times 10^3$ | 3.2 | C1/adrq+ | S117N,M133T,T126N,T131N | AF286594 |
| XJB8 | CHB | Xi'an | M/51 | −/+/−/+/+ | <500 | – | B3/adw2 | T140I | AB073826 |
| XJI33 | CHB | Xi'an | M/43 | −/−/−/+/+ | <500 | 3.2 | Ba/adw2 | Wildtype | AB073826 |
| XJQ13 | CHB | Xi'an | M/20 | −/+/−/−/+ | <500 | 5.4 | C1/adrq+ | Wildtype | AF286594 |
| XJR22 | CHB | Xi'an | F/23 | −/+/−/−/+ | <500 | 2.9 | C1/adrq+ | Wildtype | AF286594 |
| XJW2 | CHB | Xi'an | M/26 | −/+/−/−/+ | <500 | 8.9 | C1/adrq+ | Wildtype | AF286594 |
| XJP20 | CHB | Xi'an | M/29 | −/−/−/−/+ | $2.0 \times 10^3$ | – | C1/adrq+ | Wildtype | AF286594 |
| XJA34 | CHB | Xi'an | M/33 | −/−/−/−/+ | $1.5 \times 10^4$ | – | Ba/adw2 | Wildtype | AB073826 |
| QZ26 | CHB | Quanzhou | M/36 | −/+/+/−/+ | $2.0 \times 10^4$ | 7.8 | Ba/adw2 | M133T,T140I | AB073826 |
| QZ25 | CHB | Quanzhou | M/38 | −/+/−/−/+ | <500 | – | Ba/adw2 | Wildtype | AB073826 |
| X511 | ASC | Xiamen | M/43 | −/−/−/−/+ | <500 | 3.2 | C1/adrq+ | G145R | AF286594 |
| X1110 | ASC | Xiamen | M/44 | −/−/−/−/− | <500 | 2.4 | Ba/adw2 | T131N | AB073826 |
| X1710 | ASC | Xiamen | F/32 | −/+/−/−/− | <500 | 6.3 | Ba/adw2 | T131N | AB073826 |
| X3206 | ASC | Xiamen | M/26 | −/−/−/−/− | <500 | 1.2 | Ba/adw2 | Wildtype | AB073826 |
| X4236 | ASC | Xiamen | M/42 | −/+/−/−/+ | <500 | 1.5 | Ba/adw2 | Wildtype | AB073826 |

[a] "CHB" means chronic hepatitis B patients and "ASC" means asymptomatic carriers.
[b] G112stop means a stop codon mutation at the position aa 112 in HBsAg region, thrice individual PCR and sequence to confirm.

methods, MAbs or polyclonal antibody against HBsAg were used as the first or second antibody. Hence, these methods are limited for detecting HBsAg mutants. The application of available HBcrAg assays are also limited by their relatively low sensitivities and the complicated procedures (Bredehorst et al., 1985; Kimura et al., 2003). Recently, a chemiluminescence enzyme immunoassay had been developed for the detection of HBcrAg and this method shows promise for monitoring chronic HBV disease (Kimura et al., 2002; Wong et al., 2007) but its applicability in identifying HBsAg mutants remains to be seen.

In this study, a special lysis solution was used to release the PreS1 protein from the envelope, which contains the inner parts of PreS1 that cannot be detected directly unless being released from the virion and HBcAg. This lysis process enhanced dramatically the sensitivity both for PreS1 assay and for HBcrAg assay in the Combo ELISA (Fig. 1A). In dilution test, the mean detection limit of the Combo ELISA was $10^{2.9\pm0.5}$ copies/mL for HBsAg-positive sera from different genotypes, and was $10^{3.5\pm1.2}$ copies/mL for HBsAg-negative variants sera (Tables 1 and 2). Although the detection limit of PreS1 assay was observed to be about 10- to 100-fold lower than HBcrAg assay, there are cases such as XMP3 and XMP5 which exhibit poor reactivity in PreS1 assay but better performance in HBcrAg assay.

The application of Combo ELISA for detection of occult HBV infections was useful for asymptomatic HBV carriers. When 4968 healthy individuals were tested, the specificity of the assay was 99.9% (95% CI: 99.7–99.9%) and 12 were found by the Combo ELISA as positive. Among the HBsAg-negative healthy individuals, five of them were confirmed to be HBV carriers for the positive result of HBV DNA assay, and these five cases include three HBsAg variants carriers (Table 3). However, not all of the HBsAg-negative individuals could be tested for HBV DNA due to the various constraints. The occult HBV infection rate in this population cannot therefore be determined, and the sensitivity of the Combo ELISA for detecting asymptomatic occult HBV carriers also remains unclear.

The implementation of the Combo ELISA for detecting occult HBV infections was also evaluated in chronic hepatitis patients. Among 2092 hepatitis B cases, 27 occult hepatitis B cases were identified, and 10 of whom were found to carry mutations in the S gene MHR region (Table 3). The assay exhibits positive in 59.3% (16 cases) of the occult hepatitis B cases. Among the 10 "a" epitope mutants, 8 were positive in the assay and the two negative cases were also negative by q-PCR (positive by nested-PCR), which indicates the lower virus load in sera. Interestingly, a unusual premature stop codon mutation at position 112 of HBsAg (Table 3) was observed in two samples (YA112 and XJK22) with genotype Ba and C1. The two cases, with high viral load (>$10^7$ copies/mL), were negative by the conventional HBsAg assay but positive by the Combo ELISA. Similarly, stop mutation at position 69 in HBsAg had also been noted in previous report (Datta et al., 2007), and such mutations were considered to be involved in the decrease of the expression or secretion of HBsAg which is worthy of further investigation.

As demonstrated in this study, the Combo ELISA promises to be a novel indicator for HBV infections, especially for HBsAg variants. This easy-to-use assay show promise for screening larger numbers of samples.

## Conflict of interest

All authors declare there are no conflicts of interest.

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