

Title page

**HBV Core and Core-Related Antigen Quantitation in Chinese Patients with
Chronic Hepatitis B Genotype B and C Virus Infection**

Short Title: Quantitation of HBc and HBcrAg in Chinese patients

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Abbreviations: anti-HBe, antibody to hepatitis B e antigen; CLEIA, chemiluminescence enzyme immunoassay; HBeAg, hepatitis B e antigen; HBsAg, hepatitis B virus surface antigen; HBV, hepatitis B virus; HBcAg, HBV core antigen; HBcrAg, HBV core-related antigen; PCR, polymerase chain reaction

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Summary

The HBV core-related antigen (HBcrAg) and HBV core antigen (HBcAg) assays were developed for the measurement of serum HBV load. The aim of this study is to assess the clinical utility of these assays in patients infected with hepatitis B virus (HBV) in China. One hundred and ninety-three chronic hepatitis B patients were enrolled. Serum HBcrAg and HBcAg were measured by chemiluminescence enzyme immunoassay, and HBV DNA was measured by a sensitive polymerase chain reaction assay (Amplicor HBV Monitor, Roche). The data were analyzed in patients with HBV genotype B (HBV/B) and genotype C (HBV/C). The ratio of HBcrAg/HBcAg was calculated and compared between patients with and without HBeAg. The concentrations of HBcrAg and HBcAg showed significant positive correlation with the HBV DNA concentration in both HBV/B and HBV/C. The HBcrAg/HBcAg ratio was higher in patients with HBeAg than in those without HBeAg. In conclusion, the HBcrAg assay and HBcAg assay are clinically useful in viral quantitation of HBV/B and HBV/C. A combination of these assays would be a valuable tool for analyzing the clinical status of HBV infection.

Keywords: hepatitis B virus, HBV core antigen assay, HBV core-related antigen assay, antigen quantitation, HBcAg, HBcrAg/HBcAg ratio.

Introduction

Infection with hepatitis B virus (HBV) remains one of the major human infectious diseases and involves approximately 350 million people (1). In a significant proportion of cases, infection progresses to cirrhosis and liver failure as well as hepatocellular carcinoma (HCC) (2). As therapeutic advances have emerged, detailed information is required to assess the HBV replication in individual patients in clinical management.

Recently, two sensitive chemiluminescence enzyme immunoassays (CLEIA) specific for HBV were developed in our laboratory (3,4). One is an HBV core-related antigen (HBcrAg) assay that measures the serum levels of hepatitis B e antigen (HBeAg) and hepatitis B core antigen (HBcAg) simultaneously using monoclonal antibodies, and the other is an assay that measures the serum level of HBcAg. Although assessments of clinical performance relating to the HBcAg and HBcrAg assay have already been reported in Japanese patients (3-5), evaluation of these two antigen assays was not performed in patients with HBV genotype B. The aim of this study is to assess the clinical utility of the HBcAg and HBcrAg assays for measurement of HBV load in Chinese patients who are infected with genotype B or C.

Patients and Methods

Patients

Patients attending the second hospital of HeBei Medical University at Shi-Jia-Zhuang City, North China, between June and August 2001, who were persistently infected with HBV, were enrolled for the study. Serum samples obtained from 193 patients (125 male and 68 female, median age 27 years, range 5-73 years) were examined. One hundred and eighty-two patients were diagnosed as chronic HBV carriers according to the consensus diagnostic criteria of HBV infection (6). The remaining 11 patients had persistently normal alanine aminotransferase (ALT) levels, suggesting an inactive HBsAg carrier stage (6). None of the 193 patients were treated with anti-viral agents such as interferon or lamivudine. All were non-reactive for antibody to hepatitis C virus infection. All sera were stored at - 20°C until use.

HBcAg CLEIA and HBcrAg CLEIA

Concentrations of HBcAg and HBcrAg were measured in serum using the CLEIA reported previously (3,4). Briefly, 100 μ L serum was mixed with 50 μ L pretreatment solution containing 15% sodium dodecyl sulfate. After incubation at 70°C for 30 min, 50 μ L pretreated serum was added to wells coated with monoclonal antibodies against denatured HBc and HBe antigens (HB44, HB61 and HB114) and filled with 100 μ L assay buffer. The mixture was incubated for 2 h at room temperature and the wells were washed with buffer. Alkaline phosphatase-labeled monoclonal antibodies were added to the wells and incubated for 1 h at room temperature. After washing, substrate solution was added and the plate was incubated for 20 min at room temperature. The relative chemiluminescence intensity was measured, and the HBcAg concentration or the HBcrAg concentration was read by comparison to a standard curve. Recombinant HBcAg (rHBcAg: amino acids 1 to 183 of precore/core gene product) was used as a standard for the HBcAg assay and recombinant ProHBeAg (rProHBeAg: amino acids -10 to 183) was used as a standard for the HBcrAg assay, and the immunoreactivity of rProHBeAg at 10fg/mL was defined as 1 U/mL (3). The cutoff for a positive HBcAg result was 4.0 pg/mL and that for HBcrAg was 1.0×10^3 U/mL (= immunoreactivity of rProHBeAg at 10pg/mL), respectively (3,4).

The HBcrAg/HBcAg ratio was calculated in order to assess the relative amounts of HBeAg to HBcAg. The serum concentration of HBcrAg (pg/mL) was divided by that of HBcAg (pg/mL) in each sample.

Conventional HBV markers and genotyping of HBV

HBsAg, HBeAg, and anti-HBe were measured using commercially available enzyme immunoassay kits (Dinabott, Tokyo, Japan). The levels of HBV DNA in the serum samples were measured using an Amplicor HBV Monitor test (Roche Molecular Systems, Inc., Branchburg, NJ) with a detection range between 4×10^2 and 4×10^7 copies/mL. Samples with an HBV DNA level above 10^8 copies/mL were measured after dilution in HBV-negative serum. Nucleic acids were extracted from 100 μ L of sera using a Smitest Ex R&D kit (Genome Science Laboratories Co., Tokyo, Japan). HBV genotype was determined using restriction

fragment length polymorphism (7).

Statistical analysis

The Mann-Whitney's U test was utilized for quantitative data, and the Fisher's exact test was used for qualitative data. Spearman rank correlation was also employed where appropriate. Statistical analyses were done using the StatView Ver. 5.0 software package (SAS Institute Inc., Cary, NC). A *P* value of less than 0.05 was considered to be statistically significant.

Results

Genotypic distribution

Among 193 patients studied, 169 (87.6%) patients were infected with HBV of genotype C (HBV/C), 21 (10.9%) patients were infected with HBV/B, and 3 (1.5%) were infected with HBV/A. Clinical backgrounds of the patients who were infected with HBV/B and HBV/C are compared in Table 1. There were no statistical differences in clinical backgrounds, serum HBV DNA levels, serum concentrations of HBcAg, or serum concentrations of HBcrAg between the patients infected with HBV/B and HBV/C.

Correlation between HBcAg/HBcrAg and HBV DNA concentrations

The correlation between the concentrations of HBcAg and HBV DNA, and that of the concentrations of HBcrAg and HBV DNA are shown in Figure 1. The serum concentrations of HBcAg and HBV DNA correlated significantly in the patient group infected with HBV/B ($r = 0.772$, $P < 0.001$), as well as in the patient group infected with HBV/C ($r = 0.898$, $P < 0.001$). The serum concentrations of HBcrAg and HBV DNA also correlated significantly in the patient group infected with HBV/B ($r = 0.786$, $P < 0.001$), as well as in the patient group infected with HBV/C ($r = 0.865$, $P < 0.001$).

HBcrAg/HBcAg ratio

The HBcrAg/HBcAg ratio was calculated in each patient and was compared between the patients with and HBeAg and without HBeAg (Fig. 2). The data are represented in log scale. The median value of HBcrAg/HBcAg ratio was significantly higher in the patients with HBeAg (median 1.9, range; 0.9 - 4.1) than in the patients without HBeAg (median 1.6, range; 0.3 - 3.5) ($P < 0.001$).

Discussion

In this report, an attempt was made to assess the clinical utility of the HBcAg and HBcrAg assays for the measurement of HBV load in the sera from Chinese patients who are infected with HBV/B or HBV/C. In a previous study, a good quality estimation of the accuracy of the HBcrAg assay in HBV/B-infected patients could not be obtained because of the small number of patients who were infected with HBV/B. Twenty-one patients with HBV/B were enrolled in this study. As a result, a significant positive correlation was observed between the serum concentrations of HBcAg and HBV DNA, as well as HBcrAg and HBV DNA in both HBV/B- and HBV/C-infected Chinese patients. The HBcrAg assay has a high level of sensitivity, which was comparable with the real-time detection PCR (5). On the other hand, the cutoff for a positive HBcAg result was 4 pg/mL, and corresponded to approximately 4.5 log copies/mL (Fig. 1). Because an HBV level below 4 log copies/mL indicated inactive liver disease (8,9), and an HBV level higher than 5 log copies/mL is associated with active liver disease (10,11), the HBcAg assay could be valuable to differentiate chronic hepatitis B from inactive carrier state.

The HBcrAg assay detects HBcAg and HBeAg simultaneously using monoclonal antibodies that recognize both denatured HBcAg and HBeAg even in anti-HBe antibody-positive samples (3). Current commercial HBeAg assays do not detect the HBeAg/anti-HBe complex, because the epitopes of HBeAg are masked by the anti-HBe antibody (12). For the capturing HBcAg, we used HB44, HB61, and HB114 immobilized monoclonal antibodies, which were the same as in the HBcrAg assay (4). The HBcAg assay differs from the HBcrAg assay in the detection antibody, which recognizes core-specific SRRRR repeats in the C-terminal protamine-like nucleic acid binding domain and is therefore specific for HBcAg. In this report, the HBcrAg/HBcAg ratio was significantly higher in the patients with HBeAg than in the patients without HBeAg. Because the HBcrAg assay mainly reflects levels of HBeAg and HBeAg/anti-HBe complex (3), the ratio of HBcrAg/HBcAg would represent relative amounts of HBeAg to HBcAg. Should it be true, this ratio could be used as a marker that indicates a balance of HBeAg production and HBV load at some points. As HBeAg states in sera largely depend

on the HBeAg production from HBV, the mechanism of this result could be explained by the reduction of HBeAg in the sera, such as mutations in precore and core promoter region (13-15). It has also been reported that HBeAg loss occurs without mutations in the precore and core promoter region during lamivudine therapy (16). Suzuki et al. (16) conclude that the reduction in HBV level leads to reduction of HBeAg production and results in HBeAg loss during lamivudine therapy. To clarify the relationship between the reduction of HBeAg and that of HBV level during the course of HBeAg loss, the combination of the HBcAg and HBcrAg assays would be useful to distinguish natural HBeAg seroconversion from treatment-induced seroconversion. Clearly, further analysis in longitudinal studies is required and the mechanisms associated with these results remains to be explored.

In conclusion, we assessed the utility of the HBcAg and HBcrAg assays in Chinese patients with HBV/B and HBV/C. The results showed that these two HBV antigen assays are clinically useful in viral quantitation as well as HBV DNA quantitation. Using a combination of these two assays could be more useful for analyzing clinical status in patients with HBV infection.

References

1. Lee WM. Hepatitis B virus infection. *N Engl J Med* 1997;337:1733-1745.
2. Beasley RP. Hepatitis B virus. The major etiology of hepatocellular carcinoma. *Cancer* 1988;61:1942-1956.
3. Kimura T, Rokuhara A, Sakamoto Y, et al. Sensitive enzyme immunoassay for hepatitis B virus core-related antigens and their correlation to virus load. *J Clin Microbiol.* 2002;40:439-445.
4. Kimura T, Rokuhara A, Matsumoto A, et al. New enzyme immunoassay for detection of hepatitis B virus core antigen (HBcAg) and relation between levels of HBcAg and HBV DNA. *J Clin Microbiol.* 2003;41:1901-1906.
5. Rokuhara A, Tanaka E, Matsumoto A, et al. Clinical evaluation of a new enzyme immunoassay for hepatitis B virus core-related antigen; a marker distinct from viral DNA for monitoring lamivudine treatment. *J Viral Hepat.* 2003;10:324-330.
6. Lok AS, McMahon BJ. Practice Guidelines Committee, American Association for the Study of Liver Diseases. Chronic hepatitis B. *Hepatology.* 2001;34:1225-1241.
7. Mizokami M, Nakano T, Orito E, et al. Hepatitis B virus genotype assignment using restriction fragment length polymorphism patterns. *FEBS Lett.* 1999;450:66-71.
8. Lindh M, Horal P, Dhillon AP, Norkrans G. Hepatitis B virus DNA levels, precore mutations, genotypes and histological activity in chronic hepatitis B. *J Viral Hepat.* 2000;7:258-267.
9. Manesis EK, Papatheodoridis GV, Hadziyannis SJ. Serum HBV-DNA levels in inactive hepatitis B virus carriers. *Gastroenterology* 2002;122:2092-2093.
10. Lok AS, Heathcote EJ, Hoofnagle JH. Management of hepatitis B: 2000—summary of a workshop. *Gastroenterology* 2001;120:1828-1853.
11. Martinot-Peignoux M, Boyer N, Colombat M, et al. Serum hepatitis B virus DNA levels and liver histology in inactive HBsAg carriers. *J Hepatol.* 2002;36:543-546.
12. Raimondo G, Recchia S, Lavarini C, Crivelli O, Rizzetto M. Dane

- particle-associated hepatitis B e antigen in patients with chronic hepatitis B virus infection and hepatitis B e antibody. *Hepatology* 1982;2:449-454.
13. Carman WF, Jacyna MR, Hadziyannis S, et al. Mutation preventing formation of hepatitis B e antigen in patients with chronic hepatitis B infection. *Lancet* 1989;2:588-591.
 14. Okamoto H, Yotsumoto S, Akahane Y, et al. Hepatitis B viruses with precore region defects prevail in persistently infected hosts along with seroconversion to the antibody against e antigen. *J Virol* 1990;64:1298-1303.
 15. Okamoto H, Tsuda F, Akahane Y, et al. Hepatitis B virus with mutations in the core promoter for an e antigen- negative phenotype in carriers with antibody to e antigen. *J Virol* 1994;68:8102-8110.
 16. Suzuki F, Tsubota A, Arase Y, et al. HBe antigen loss during lamivudine therapy is not caused by mutations in precore and core promoter genes in patients with chronic hepatitis B. *J Med Virol*. 2003;70:355-360.

Figure Legends

Fig. 1: The degree of correlation between the concentrations of HBcAg and HBV DNA, and those of HBcrAg and HBV DNA. 1A and 1B represent the correlation between the concentrations of HBcAg and HBV DNA in the sera from patients infected with HBV/B and HBV/C, respectively. 1C and 1D represent the correlation between the concentrations of HBcrAg and HBV DNA in the sera from patients infected with HBV/B and HBV/C, respectively. Closed circles indicate data from HBeAg positive sera, while open circles indicate data from HBeAg negative sera. HBV DNA levels were determined by the Amplicor HBV Monitor test (Roche Molecular Systems, Inc., Branchburg, NJ). Broken lines indicate the lower cutoff of the assays.

Fig. 2: HBcrAg/HBcAg ratios in relation to hepatitis B e antigen (HBeAg) status. Data are represented by a box-plot showing the 25th percentile, median, and 75th percentile as vertical box. Tick marks above and below the box indicate the 90th and 10th percentiles (log representation). Each outlier data point is shown as closed circle.

Table 1. Background Characteristics of Patients Infected with HBV of Genotype B and Genotype C

Features	Genotype B (n = 21)	Genotype C (n = 169)	<i>P</i> value
Age (years) ^a	22 (9-65)	27 (5-73)	NS
Men ^b	12 (57.1%)	111 (65.7%)	NS
HBeAg positivity ^b	16 (76.2%)	102 (60.4%)	NS
ALT (U/L) ^a	50 (21-105)	47 (10-2100)	NS
HBV DNA (Log copies/mL) ^{a †}	8.7 (4.4-9.4)	7.5 (3.0-9.4)	NS
HBcAg (Log U/mL) ^a	6.3 (2.2-7.4)	5.7 (1.9-7.5)	NS
HBcrAg (Log U/mL) ^a	8.3 (2.9-8.9)	8.0 (2.5-9.0)	NS

^a Data are expressed as median (range).

^b Data are expressed as positive number (%).

†HBV DNA was measured by an Amplicor HBV Monitor test (Roche Molecular Systems, Inc., Branchburg, NJ).