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ORIGINAL ARTICLE

Correlation of HBV Core-Related Antigen with Conventional Serologic Indicators of Hepatitis B and Disease Stage

Yinmiao Wang [1], Xiaohua Jin [1], Qunxin Peng [1], Hang Run [2], Yanhui Song [1]

[1] *Center of Clinical Laboratory, The First Affiliated Hospital of Soochow University, Soochow University, China*
[2] *The Institutes of Biology and Medical Sciences, Soochow University, China*

SUMMARY

Background: **Hepatitis B caused by hepatitis B virus (HBV) infection is a serious global public health issue. Currently, serological indicators serve as important markers for the diagnosis of hepatitis B. It has been found that HBV core-related antigen (HBcrAg) correlates well with intrahepatic cccDNA, intrahepatic HBV DNA, serum HBV DNA, and hepatitis B e antigen (HBeAg). To provide a more reliable basis for the diagnosis and treatment of hepatitis B, we explored the correlation between HBcrAg and conventional serologic testing indicators and disease staging.**
Methods: **Five hundred forty-two patient serum samples were collected at the First Affiliated Hospital of Soochow University from November 2021 to March 2022. The serum HBcrAg was measured by the magnetic particle chemiluminescence method in addition with other serum indicators.**
Results: **HBcrAg statistically correlated with HBV DNA level ($r = 0.655$, $p < 0.001$) and HBeAg level ($r = 0.945$, $p < 0.001$. The mean HBcrAg levels in the immune-tolerant and immune-clearance phases were significantly higher than those in the immunologic-control phase and the reactivation phase. This study demonstrated that serum HBcrAg positively correlated with serum HBV DNA and HBeAg. Even in cases where HBV DNA and HBeAg are negative, there is still a higher positivity rate of HBcrAg in hepatitis B patients.**
Conclusions: **HBcrAg is a reliable serum marker to avoid underdiagnosis of occult HBV infection.**
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Correspondence:
Dr. Yanhui Song
Center of Clinical Laboratory
The First Affiliated Hospital of Soochow University
Clinic Bldg. Rm 201
899 Pinghai Road
Suzhou, 215031
China
Phone/Fax: + 86 512-67780458
Email: syh430071@163.com

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INTRODUCTION

Hepatitis B caused by hepatitis B virus (HBV) infection is a serious global public health issue endangering human beings. The prevalence of hepatitis B surface antigen (HBsAg) positivity is 5% - 6% globally. According to WHO statistics, there are 296 million people with chronic HBV infection, which led to approximately 820,000 deaths annually [1]. Now, 70 million people live with HBV infection in China [2,3]. Patients with HBV infection are susceptible to developing cirrhosis, liver failure, and hepatocellular carcinoma (HCC). Therefore, the diagnosis and treatment of hepatitis B is crucial.

Currently, the traditional testing indicators for hepatitis B are serological indicators such as HBsAg, anti-hepatitis B surface antibody (anti-HBs), hepatitis B e antigen (HBeAg), anti-hepatitis B e antibody (anti-HBe), anti-hepatitis B core antibody (anti-HBc), HBV DNA, ALT enzyme, etc. The positivity of these serological indicators is an important criterion for the hepatitis B diagnosis. Serological negativity conversion of HBeAg and HBV DNA is also commonly used as the endpoints of antiviral treatment in clinics [4,5]. The percentage of HBeAg-negative chronic hepatitis B patients has increased in recent years due to the antiviral therapy and the aging of the HBV-infected population [6]. Therefore, a single test of traditional serologic markers is prone to underdiagnosis and some hepatitis B patients still progress to cirrhosis, hepatocellular carcinoma, and other illnesses after HBeAg and HBV DNA conversion. The quantitative detection of intrahepatic covalently closed circular DNA (cccDNA) or HBV integrated DNA via liver biopsy provides a more accurate way to evaluate the antiviral efficacy and treatment endpoints [7], but it is an invasive detection. HBV core-related antigen (HBcrAg) is a denatured mixture consisting of HBeAg, hepatitis B core antigen (HBcAg), and a 22-kDa precore protein (p22cr) [8]. HBeAg, HBcAg, and p22cr are all products of the precore/core gene and share 149 amino acid residues [9]. In 2002, HBcrAg was first reported as a target in the development of a sensitive enzyme immunoassay specific for hepatitis B core antigen (HBcAg) and HBeAg [10]. It has recently been found that HBcrAg correlates well with intrahepatic cccDNA, intrahepatic HBV DNA, serum HBV DNA, and HBeAg [11], and to a lesser extent with HBsAg [12]. It also can be detected when serum HBV DNA and HBeAg are negative [11,12]. In this study, we analyzed the HBcrAg of 542 HBV infected patients and its correlation with HBV DNA, HBeAg, and disease stage. The combination of HBcrAg with other traditional serum markers may provide new diagnostic and therapeutic options for hepatitis B patients.

MATERIALS AND METHODS

Ethics

This study was approved by the Ethics Committee of the First Affiliated Hospital of Soochow University and conducted in accordance with the ethical standards of the Helsinki Declaration. Written informed consents were obtained from all participants who volunteered for the study. All data were analyzed anonymously.

Samples

A retrospective study was conducted according to the principle of informed consent. Based on this, 542 plasma samples were obtained from HBV-infected patients who visited the First Affiliated Hospital of Soochow University from November 2021 to March 2022, including 346 males, and 196 females, aged 15 - 87 years, with a mean age of 42.48 ± 13.16 years. All patients had been excluded from hepatitis A, C, D, and E co-infection or overlapping infection, and the HBV infection was based on HBsAg positive testing except one patient who had hepatitis B over 20 years. This patient was negative for HBsAg testing but positive for serum HBV DNA. All study subjects gave informed consent.

Serum HBcrAg quantification

HBcrAg was detected by the magnetic particle chemiluminescence method. Hepatitis B Virus Core-Associated Antigen Determination Kit (Chemiluminescence Immunoassay) was the reagent used in the test, which was performed on a Myriad Chemiluminescence Immunoassay Analyzer CL-6000 I. If the concentration of HBcrAg exceeded the upper limit, then the samples were diluted 100-fold for quantification. The result was considered positive if it was > 2.6 log U/mL.

Serum HBV DNA measurements

The fluorescence quantitative polymerase chain reaction (qPCR) method was used, the reagents were purchased from Changsha Shengxiang Bio-technology Co., Ltd, and the instrument was a LightCycler 480 PCR amplifier from Roche, Switzerland. The quantitative detection value of HBV DNA > 1.0×10^3 IU/mL was regarded as a positive result.

Serum HBeAg measurement

HBeAg was detected using chemiluminescence microparticle immunoassay, Abbott Ireland Diagnostics provided the test kit, which was used exactly as instructed. The results were read and recorded, and the result of the test ≥ 1.0 S/CO was regarded as positive.

Serum ALT measurements

The test was performed by Siemens ADVIA 2400 automatic biochemical analyzer, and Ningbo Meikang Biotechnology Co. supplied the reagents. Women's normal ALT values range from 7 to 40 U/L, whereas men's normal ALT values range from 9 to 50 U/L.

Disease staging

Referring to the "Guidelines for the prevention and treatment of chronic hepatitis B (2022 version)", patients were staged according to the results of the Hepatitis B serologic test, HBV DNA, and serum ALT levels. Patients with missing serum ALT data were not included in the staging. Patients were divided into four periods: immune-tolerant, immune-clearance, immune-control, and reactivation periods as shown in Table 1.

Statistical analysis

GraphPad Prism9 statistical software was used to visualize the correlation between serum HBcrAg, serum HBV DNA, serum HBeAg, and disease stage by linear analysis and chi-squared test, and $p < 0.05$ was considered statistically significant.

Table 1. Phases of chronic HBV infection.

Phases	HBeAg-positive chronic HBV infection (immune tolerance phase, chronic HBV carrier)	HBeAg-positive CHB (immune clearance phase, immune active phase)	HBeAg-negative chronic HBV infection (immune control phase, inactive HBsAg carrier)	HBeAg-negative CHB (reactivation phase)
HBsAg (IU/mL)	> 1×10^4	+	< 1×10^3	+
HBeAg	+	+	–	–
HBV DNA (IU/mL)	> 2×10^7	+	–	+
ALT	< ULN	elevated (persistently or repeatedly)	< ULN	elevated (persistently or repeatedly)
Liver histology	none/minimal necroinflammation and fibrosis	obvious necroinflammation and/or fibrosis	minimal/mild inflammation but different degrees of fibrosis	obvious necroinflammation and/or fibrosis

HBV - hepatitis B virus, HBsAg - hepatitis B surface antigen, HBeAg - hepatitis B e antigen, ALT - alanine aminotransferase, CHB - chronic hepatitis B, ULN - upper limit of normal.

Table 2. Relationship between HBV DNA and HBcrAg positivity rate.

	HBcrAg		HBeAg	
HBV DNA positive	positive	negative	positive	negative
348	285 (81.9%)	63 (18.1%)	141 (41.5%)	207 (58.5%)
HBV DNA negative	positive	negative	positive	negative
194	113 (58.2%)	81 (41.8%)	32 (16.5%)	162 (83.5%)

Table 3. Relationship between HBeAg and HBcrAg positivity rate.

	HBcrAg	
HBeAg positive	positive	negative
173	173 (100%)	0
HBeAg negative	positive	negative
369	225 (61.0%)	144 (39.0%)

RESULTS

Correlation analysis of serum HBcrAg and serum HBV DNA levels

The HBcrAg and HBV DNA level was measured in 542 collected samples. It was found that HBcrAg correlates with HBV DNA in logarithmic values using correlation analysis and chi-squared test ($r = 0.655$, $p < 0.001$) (Figure 1A). According to HBV DNA level, these samples can be divided into four groups: Group A ($\leq 10^3$ IU/mL), Group B (10^3 - 10^5 IU/mL), Group C (10^5 - 10^7 IU/mL), and Group D ($\geq 10^7$ IU/mL) in our study. For the HBcrAg level detection, Group A's median log value was 2.821 IU/mL, Group B's median log value was 3.281 IU/mL, Group C's median log value was 5.376 IU/mL, and Group D's median log value was 8.415 IU/mL. In terms of HBcrAg, although the mean log value of Groups B was close to Group A, there was a signi-

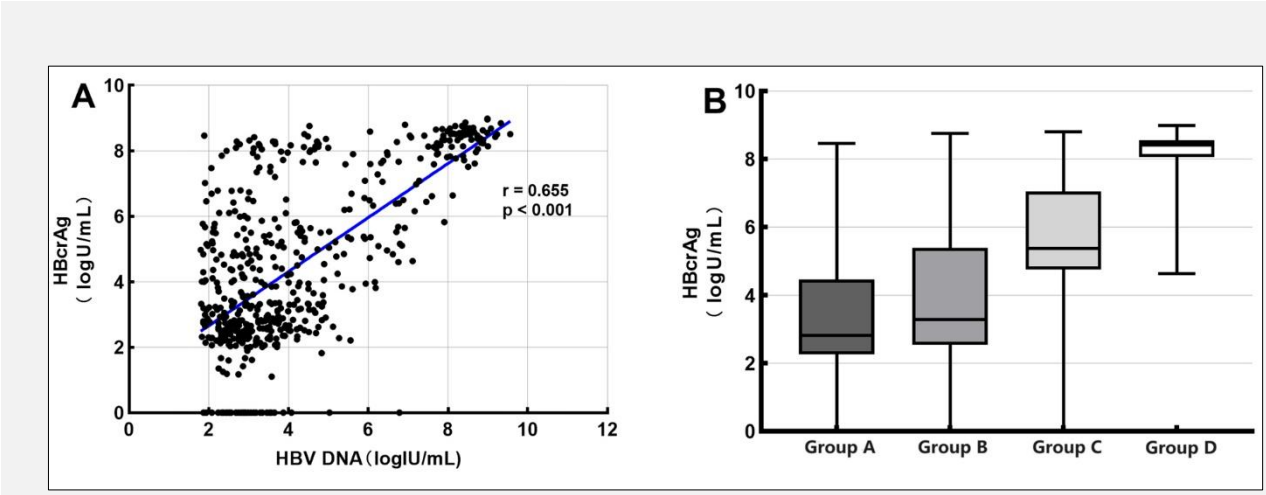

Figure 1. Serum HBcrAg level correlation with serum HBV DNA level.

A - Dot plot analysis of the correlation between serum HBcrAg level and serum HBV DNA, B - The HBcrAg levels in different groups defined by HBV DNA level. The data was shown as median ± SD.

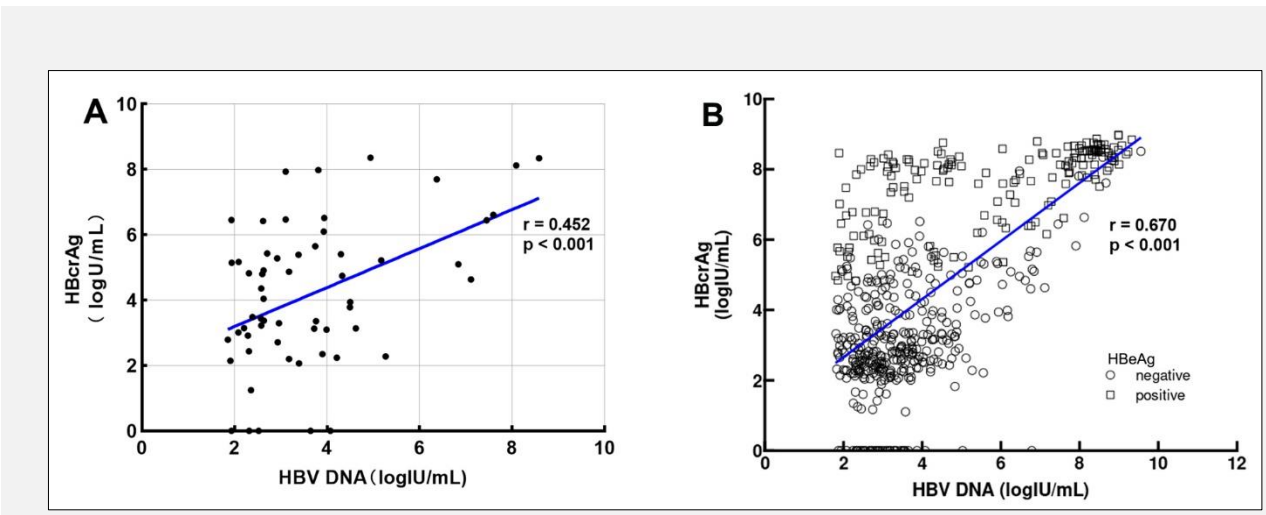

Figure 2. The correlation between serum HBcrAg and HBV DNA in medicated or unmedicated groups.

A - The correlation between serum HBcrAg and serum HBV DNA in the medicated group, B - The correlation between serum HBcrAg and serum HBV DNA in the unmedicated group.

ficant difference ($p < 0.001$). It was clear that the HBcrAg level increased with HBV DNA level, especially in Groups B, C, and D (Figure 1B).

The sample data also can be divided into two groups based on the patient's medication status: the explicit medication group and the unspecified group. When comparing the correlation between HBcrAg and HBV DNA levels of these two groups, the correlation coefficient of the explicit medication group is lower than the unspecified group's correlation coefficient ($r = 0.452$, $p < 0.001$ vs. $r = 0.670$, $p < 0.001$, Figure 2A and 2B).

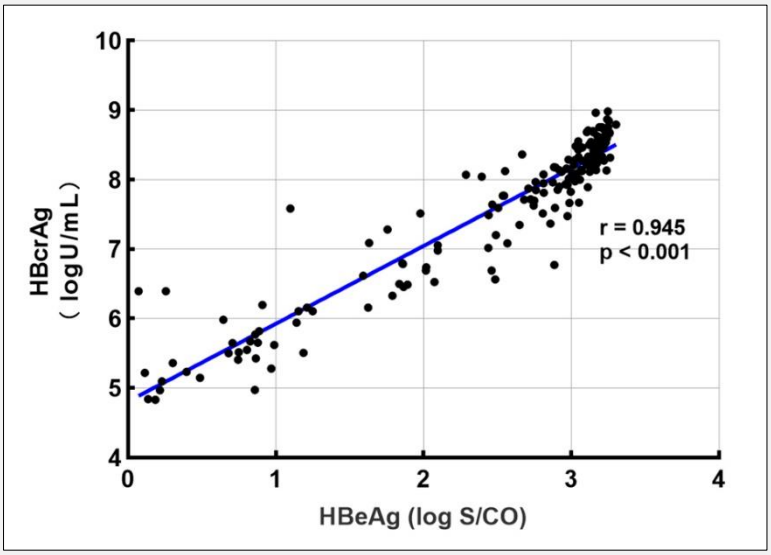

Figure 3. The correlation between serum HBcrAg and serum HBeAg.

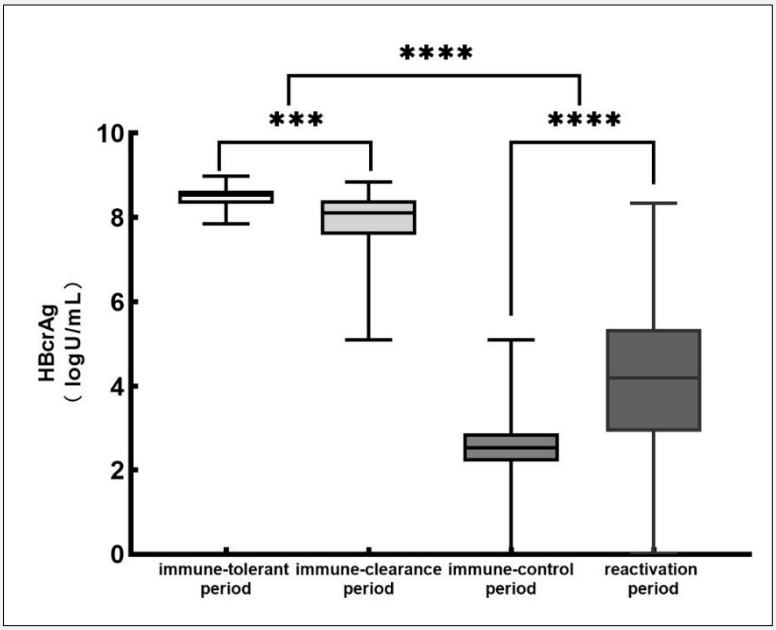

Figure 4. The distribution of serum HBcrAg levels at different disease stages.

The data was shown as median ± SD. * - p value < 0.001; **** - p value < 0.0001.**

Serum HBcrAg is more responsive than HBV DNA for viral replication activity

The total number of HBV DNA positive samples was 348, with 285 HBcrAg positive test (81.9%) and 141 HBeAg positive test (41.5%). The total number of HBV DNA negative samples was 194 cases, the number of positive HBcrAg tests was 113 (58.2%), and the number of HBeAg positive cases was 32 (16.5%). This confirmed that HBcrAg positivity was more sensitive than HBeAg, regardless if the sample was HBV DNA positive or negative (Table 1).

Serum HBcrAg is more responsive than HBeAg for viral replication activity

The clinical HBeAg positive index suggests that the virus is actively replicating and highly infectious [13]. We then analyzed the correlation of serum HBcrAg with HBeAg which could further prove the clinical value of HBcrAg. There were 173 HBeAg-positive samples, and these samples were 100% positive for HBcrAg (Table 2). A strong correlation in logarithmic values was obtained between HBeAg and HBcrAg serum levels ($r = 0.945$, $p < 0.001$) (Figure 3). Moreover, of the 369 HBeAg-negative samples, the number of HbcrAg-positive cases was 225 with a positive rate of 61.0% (Table 2). Above all, results confirmed that serum HBcrAg was more responsive than HBeAg for viral replication activity.

HBcrAg varies in different stages of chronic hepatitis B

According to the “Guidelines for the prevention and treatment of chronic hepatitis B” [2], and the traditional serological indicators of Hepatitis B, HBV DNA, and serum ALT enzyme, samples from 542 patients were also classified into four different periods: immune-tolerant, immune-clearance, immune-control, and reactivation periods. The data of HBcrAg were classified according to these four periods, and the results were shown in Figure 4. In immune tolerance and immune clearance periods, the mean HBcrAg level was significantly higher than that in the immune control and reactivation periods ($p < 0.0001$); the mean HBcrAg level was higher in the immune tolerance period than in the immune clearance period ($p < 0.001$); in the active period compared to the immune control period, it still was greater ($p < 0.0001$). These levels suggested that HBcrAg might be a valuable index to distinguish the different progression stages of chronic hepatitis B.

DISCUSSION

This cross-sectional study aims to investigate the correlation of HBcrAg with the serum markers and the differences in disease stages. Previous research has shown that HBcrAg is strongly correlated with intrahepatic cccDNA and intrahepatic HBV DNA [14]. Our findings are in line with Seto et al. [15] demonstrating a high association between serum HBcrAg and serum HBV DNA. We found that the level of HBcrAg increased with HBV DNA level, indicating a positive correlation especially in Group B, C, and D. It might serve to reflect the degree of viral replication. When the patients were divided into groups based on the medications they were taking, it was shown that the correlation between HBV DNA and HBcrAg had a strong correlation in the unspecified group, whereas it decreased in the explicit medicated group. The cause may be that the secretion of HBV particles containing HBcAg and HBV DNA depends on reverse transcription which is suppressed by the antiviral drugs while antiviral therapy has no direct effect on HBeAg production and secretion [15]. In Table , HBcrAg has a high detection rate compared with HBeAg detection rate regardless of HBV DNA positivity. The reason might be that although the antiviral therapy results in serum HBV DNA negativity, the infected hepatocytes containing cccDNA will still secrete HBeAg, HBcAg, and p22cr proteins [11, 15,16].

In our results, HBcrAg-positive samples have a strong correlation with HBeAg since the HBcrAg kit detects the pre-core/core proteins of HBeAg, HBcAg, and p22cr at the same time. It was confirmed by the fact that all the HBeAg-positive samples are HBcrAg positive as shown in Table . Even more, HBeAg-negative samples still have a high HBcrAg positive rate. HBeAg negativity may be caused by mutations in the pre-C region of the HBV viral gene, which also affects the formation of the p22cr protein but not the production of HBcAg [17-19]. Therefore, the detection of HBcrAg is more conducive to evaluate the patient's response to treatment and determine the end of therapy. By combining the test with HBV DNA and hepatitis B serologic test, patients with HBV DNA or HBeAg conversion can avoid delayed treatment or premature medication discontinuation as well as the progression of some serious conditions like cirrhosis and hepatocellular carcinoma.

With a single HBeAg serum indicator, it is hard to determine the disease stage of hepatitis B patients because both immune tolerance and immune clearance periods are HBeAg positive, while the immune-controlled and reactivation periods are HBeAg negative. In line with the previous findings [20], the HBcrAg level in our study has distinct levels in different stages. It would be very valuable for doctors to be able to distinguish disease stages based on the difference between the HBcrAg level in HBeAg-positive group (immune tolerance and immune clearance) or HBeAg-negative group (immune-controlled and reactivation). However, in addition to HBeAg level, more accurate thresholds of HBcrAg should be established for distinguishing these stages.

Our study still has some limitations. First, as a clinical trial, we were unable to examine the connections between HBcrAg and intrahepatic cccDNA, intrahepatic HBV DNA, and HBV RNA. Second, due to the lack of information on liver pathology and the lack of HBsAg value of some samples whose HBsAg value is over the detection limit, HBsAg and liver pathology were not referenced during staging. The lack of exact data for HBsAg also prevented analysis of the associations be-

tween HBcrAg and HBsAg. Third, this experiment is a cross-sectional study, its arguments are not as good as longitudinal studies. HBcrAg could not be used to evaluate drug efficacy and discontinuation time, predict discontinuation relapse and the occurrence of hepatocellular carcinoma in the absence of follow-up investigation. In conclusion, the newly discovered serum marker HBcrAg is an indicator with obvious advantages. It has a strong correlation with serum markers, especially in patients who are still negative for HBV DNA and HBeAg, and is more easily accessible when compared with invasive tests for intrahepatic marker detection. Combined testing with conventional serum markers can avoid underdiagnosis of occult HBV infection and enable hepatitis B patients to benefit from closer viral monitoring and efficacy assessment.

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Declaration of Interest:
The authors declare they have no financial/commercial conflicts of interest.

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