DIAGNOSING DIFFERENT STAGES OF HEPATITIS B INFECTION USING A COMPETITIVE POLYMERASE CHAIN REACTION ASSAY

H Changotra, A Dwivedi, AK Nayyar, *PK Sehajpal

Abstract

Purpose: Different stages of hepatitis B virus (HBV) infection can be defined by serum HBV DNA levels. This study attempts to (1) investigate serum HBV DNA levels in inactive carriers and patients with chronic HBV (CHB) infection and (2) define cut-off value between inactive carriers and HBeAg (precore antigen of HBV) negative CHB patients in Indian population. Methods: One hundred and forty samples encompassing 42 inactive HBsAg carriers and 98 CHB patients (53 HBeAg-positive and 45 HBeAg-negative) were analysed. Serum HBV DNA levels were determined employing an in-house competitive polymerase chain reaction (cPCR) assay. Results: The HBeAg-positive patients were found to have the maximum median HBV DNA load, which was significantly higher than the HBeAg-negative ones (median; 1.25 × 10⁸ vs. 2.30 × 10⁸ copies/mL; P < 0.05). Interestingly, the latter group has significantly higher HBV DNA levels than the inactive carriers (median; 2.30 × 10⁸ vs. 4.28 × 10⁹ copies/mL; P < 0.05). The 2.5 × 10⁴ copies/mL HBV DNA levels were optimal for discriminating CHB patients (HBeAg-negative) from inactive carriers with 75.6 and 78.6% sensitivity and specificity, respectively. Conclusions: Despite the extensive overlapping of HBV DNA levels in inactive carriers and HBeAg negative CHB patients, 2.5 × 10⁴ copies/mL is the most favourable cut-off value to classify these individuals and would be imperative in the better management of this dreadful disease.

Key words: Chronic hepatitis B, HBeAg, HBsAg, HBV DNA, inactive carriers, quantitation

Approximately, one-third of the world population has serological evidence of the past or present Hepatitis B virus (HBV) infection resulting in 400 million chronically infected people.¹ This could lead to a variety of clinical outcomes ranging from an apparently healthy asymptomatic carrier state to acute or chronic liver disease, including cirrhosis and hepatocellular carcinoma.¹,² Persistent presence of hepatitis B surface antigen (HBsAg) for at least six months defines the chronic hepatitis B (CHB) carrier state. Conventionally, presence of secretory version of a HBV core protein, the e antigen (HBeAg), is associated with high viral load and serves as a marker for viral replication.³ HBeAg seroconversion (HBeAg negative and anti-HBe) is associated with liver disease remission and marks the transition from chronic hepatitis B to the asymptomatic HBsAg carrier state, which does not show the clinical symptoms of the disease. At the time of HBeAg seroconversion, a small percentage of patients continue to show raised alanine aminotransferase (ALT) and serum HBV DNA levels.³ This group of patients is called as HBeAg negative CHB which continues to have liver damage but due to frequent changes of ALT levels, becomes difficult to differentiate from inactive carriers.¹,⁴ In the past decade, detection and quantification of HBV infection in patients has drastically evolved due to the advent of molecular tools. This has immensely helped in understanding the pathogenesis and the natural course of HBV infection³ and simultaneously, raised new predicaments and clinically relevant questions.

To address some of the issues, a research workshop held at National Institute of Health (NIH), USA, on hepatitis B recommended the use of a new term “inactive HBsAg carrier state” instead of healthy asymptomatic carriers. Additionally, it was proposed that this class of HBV carriers is better defined by an arbitrary HBV DNA level below 10⁵ copies/mL of the serum² and the patients having levels more than this value should undergo antiviral treatment (AVT), irrespective of the HBeAg status contrary to earlier belief. Following this, various investigators have attempted to validate the proposed cut-off limits for differentiating these two groups of carriers and found that a lower threshold value might be more relevant in their populations.⁶,⁷ Such a study turns out to be imperative in a country like India where no such data exists and the burden of this virus is second highest in the world.⁸ The present study attempts to fill this void.

Materials and Methods

Patients, liver function tests and viral markers

Serum samples from the HBV infected patients as well as normal healthy individuals were collected from the
Guru Nanak Hospital, Amritsar, Ram Saran Das Kishori Lal Charitable Trust Hospital, Amritsar and Dr. Ram Manohar Lohia Hospital, New Delhi, India. All the patients or their legal representative gave informed consent. The samples included in the study met the following criteria: Age between 16 and 75 years, hepatitis B surface antigen positive for at least six months, no history of malignancy, alcohol intake, and human immunodeficiency virus (HIV) or hepatitis C virus infection and had not undergone antiviral treatment during the past six months. During the past six months, the serum alanine aminotransferase (ALT) levels were persistently within and at least once above the normal range among the inactive and chronic carriers, respectively. The latter category of patients was further classified based on core antigen (HBeAg) as HBeAg-negative and HBeAg-positive CHB patients, thus resulting in three groups i.e., inactive carriers (42; HBeAg-negative; normal ALT levels), HBeAg-negative CHB patients (45; HBeAg-negative; ALT elevated) and HBeAg-positive CHB patients (53; HBeAg-positive; ALT elevated).

Collected serum samples were screened for the presence of HBsAg and HBeAg serological markers using commercially available kits (Abbott Laboratories, North Chicago, IL). Serum ALT levels were measured using a commercial kit (Bayer Diagnostics, USA) for which the upper normal limit is 40 IU/l.

HBV DNA isolation

Fifty microlitre of microwaved serum diluted with 350 µL of TE buffer (10 mM Tris, 1mM EDTA; pH 8.0), was incubated at 56°C for two hours in the presence of proteinase K (1 mg/mL) and SDS (0.66%). Subsequently, the proteins were removed employing 3.4 mM ammonium acetate at room temperature. The DNA precipitated from the supernatant with 2.5 volumes of chilled ethanol (95%) in the presence of 2.5 µg glycogen (Ambion, Austin, Texas) was then washed with 70% ethanol, air-dried and finally dissolved in 20 µL of TE buffer. The DNA solution was stored at -20°C until further use.

Quantitative detection of HBV DNA

HBV DNA levels were quantified in all the collected 140 samples using an S gene based novel in-house competitive-polymerase chain reaction (cPCR) assay.12 This assay enables the quantitative detection of HBV DNA ranging from 10^3 through 10^12 copies/mL of HBV DNA from human serum. Briefly, a constant amount of unknown HBV DNA was co-amplified with known concentration of competitor DNA construct (mimic). This resulted in the amplification of two products; one corresponding to target (300 bp) and other to competitor (230 bp) employing the same primer pair. The amplification mixture (30 µL) contained 3 U of Taq polymerase (Bangalore Genie, India), 200 µM of dNTPs mixture (dTTP, dCTP, dGTP and dTTP), 0.2 µM of each primer (FW1; 5’-GGT ATG TTG CCC GTT TGT CC-3’ and RW1; 5’-CCC AAT ACC ACA TCA AT-3’), 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 1.5 mM MgCl_2 and 0.01% gelatin. The DNA sample and mimic were added in the last. The reaction was amplified in a programmable thermal cycler (MJR Research Products, USA). The amplification profile consisted of initial 3 minute denaturation at 94°C, followed by 35 cycles of (1) denaturation at 94°C for 30 seconds, (2) annealing at 57°C for 30 seconds and (3) extension at 72°C for 30 seconds with a final extension at 72°C for 3 minutes. The PCR products were electrophoresed in 2.5% agarose gel inTris-Acetate-EDTA (TAE) buffer; pH 8.0. The ethidium bromide stained gel was visualised and photographed using UV transilluminator and digital imaging system (Ultralum Inc, USA). Following agarose gel electrophoresis, the PCR products of two targets were densitometrically quantified (ImageJ1.33u, NIH, USA; http://rsb.info.nih.gov/ij) and the point of equivalence was determined leading to the quantification of serum HBV DNA levels.13

Statistical analysis

Statistical analysis was carried out using the SPSS ver. 7.5 for windows software (SPSS Inc, Chicago, IL, USA). The HBV DNA levels and other clinical/laboratory parameters were expressed as the median value and range. The differences between the groups were analysed using the Chi-square test and ANOVA. Receiver operator curve (ROC) curve was used to determine the cut-off levels of HBV DNA that differentiated HBeAg-negative CHB patients and inactive HBsAg carriers. Results were considered statistically significant at P < 0.05.

Results

The demographic and clinical data of the three studied groups were compared with respect to sex, age, ALT and HBV DNA levels (Table). HBV DNA levels were estimated employing cPCR assay as described in the section, materials and methods. Figure 1 depicts a representative agarose gel electrophoresis picture of the co-amplified products in case of an inactive carrier (A) and an HBeAg-negative CHB patient (B) and its computational analysis (C) for the calculation of viral load, which was observed to be 7.55 × 10^8 copies/mL in (A) and 2.27 × 10^8 copies/mL in (B).

The three studied groups had predominantly male population with similar median age. ALT levels differed statistically in these groups (ANOVA; P < 0.05). Baseline serum HBV DNA levels among the HBeAg-negative patients were significantly lower than the HBeAg-positive patients (median; 2.30 × 10^5 vs. 1.25 × 10^6 copies/mL; P < 0.05) and significantly higher than the inactive carriers (median; 2.30 × 10^5 vs. 4.28 × 10^3 copies/mL; P < 0.05).

HBV DNA levels overlapped extensively between HBeAg-negative CHB patients and inactive carriers; despite this the levels between the two groups differed
The optimal HBV DNA cut-off level on Receiver Operator Curve discriminating HBeAg-negative CHB and inactive carriers was 2.50 × 10^4 copies/mL, with 75.60% sensitivity and 78.60% specificity (Fig. 2). Nine out of forty-two (21.42%) of inactive carriers were above and 24.44% (11/45) of HBeAg-negative CHB patients were below this value. However, 1/42 (2.38%) of the inactive carriers and 27/45 (60.00%) HBeAg negative CHB were respectively, above and below the NIH cut-off value of 10^5 copies/mL.

### Table: Baseline characteristics of chronic hepatitis B patients, HBeAg-positive, HBeAg-negative patients and inactive carriers

<table>
<thead>
<tr>
<th>Patient characteristics</th>
<th>Chronic hepatitis B patients</th>
<th>Inactive HBsAg carriers (42)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (year)</td>
<td>41.5 (17-70)</td>
<td>45.5 (16-75)</td>
<td>NS</td>
</tr>
<tr>
<td>Sex (M/F)</td>
<td>32/21</td>
<td>28/17</td>
<td>NS</td>
</tr>
<tr>
<td>HbsAg</td>
<td>Positive</td>
<td>Positive</td>
<td></td>
</tr>
<tr>
<td>HbeAg</td>
<td>Positive</td>
<td>Negative</td>
<td></td>
</tr>
<tr>
<td>ALT (IU/l)</td>
<td>90 (30-370)</td>
<td>76 (18-220)</td>
<td>&lt;0.05^a</td>
</tr>
<tr>
<td>HBV DNA levels (copies/ml)</td>
<td>1.25 × 10^8</td>
<td>2.30 × 10^5</td>
<td>&lt;0.05^a</td>
</tr>
</tbody>
</table>

NS - not significant; ^ANOVA between three groups

![Figure 1](image1.png)

**Figure 1:** (A) and (B) Representative agarose gel electrophoresis picture of cPCR amplified products for the calculation of HBV DNA load in the serum of an inactive carrier (A) and an HBeAg-negative CHB patient (B). Lane M represents 100 bp marker and other lanes represent varying amount of competitor; C (amount is shown at the upper side of the gel picture) co-amplified with constant unknown amount of Target HBV DNA; T from both the categories mentioned. (C) Computational analysis of the data obtained from (A) and (B) plotted as ratio of log of HBV DNA to competitor vs. log of competitor (+DNA from inactive carrier* DNA from HBe Ag-negative CHB patient)

![Figure 2](image2.png)

**Figure 2:** Receiver Operator Curve (ROC) for calculating the HBV DNA level differentiating HBeAg-negative CHB from inactive carriers using Analyse-it (England, UK). The cut-off value was set at 2.5 × 10^4 copies/mL with 75% sensitivity and 78.6% specificity

**Discussion**

HBV-infected patients show variety of clinical symptoms ranging from an apparently healthy inactive carrier state to fulminant hepatitis or chronic liver disease, including cirrhosis and hepatocellular carcinoma. Inactive carriers have lower risk of progression to liver cirrhosis as well as to liver cancer compared to chronic hepatitis B patients with the latter benefiting from the anti-viral treatment. One of the most common ironies in case of chronic hepatitis B patients is the differentiation between HBeAg-negative CHB cases from the inactive carriers as they share their serological profile. Viral load quantification plays a vital role in the better management of this dreadful pathogen as it is helpful in defining the state of infection, designing drug regimen as well as in monitoring antiviral treatment. Consequently, the present study was attempted to define the serum HBV DNA cut off levels differentiating the three studied states of HBV infection relating to Indian population.

In our study, the observed median HBV DNA levels in different stages of HBV infection were different from...
the ones reported earlier on other populations of the world.\textsuperscript{5,9} However, the median HBV DNA level for the HBeAg-positive patients was statistically higher than the HBeAg-negative ones and similar trend was observed between the HBeAg-negative CHB patients and the inactive carriers (Table). Various studies on different populations of the world suggest that a lower cut-off value appears to be a better marker for differentiating HBeAg-negative CHB patients from the inactive carriers\textsuperscript{6-9} as compared to NIH recommended value of 10\textsuperscript{5} copies/mL. In the present study also, we found 2.5 × 10\textsuperscript{4} copies/mL (Fig. 2) as a better limit to differentiate the said categories of individuals. On categorising the patients according to NIH guidelines, 60.0\% of HBeAg-negative-CHB patients were below and 2.4\% of inactive carriers were above the cut off value. Similarly, according to 2.5 × 10\textsuperscript{4} copies/mL value, 24.4\% of HBeAg-negative CHB and 21.4\% of inactive carriers were below and above this value, respectively. The data of inactive carriers in this study is in accord with earlier report by Martinot-Peignoux \textit{et al.},\textsuperscript{19} according to which 98\% of the inactive carriers have HBV DNA levels below the NIH cut-off level and 97\% of them were found to be stable during follow-up. HBV DNA levels of HBeAg-negative CHB patients could not be compared, as they were not included in their study. However, according to a study by Chu \textit{et al.},\textsuperscript{6} NIH recommended value excluded 45.0\% of HBeAg-negative CHB patients in their population. Similarly, Hoe \textit{et al.},\textsuperscript{7} observed while studying Korean population, that NIH value exclude 40.4\% HBeAg-negative CHB patients and additionally, 20.0\% of the inactive carriers were above this value. Evidently, HBV DNA levels in Indian HBsAg-positive population are lower in both the cases. These variations may be attributed to ethnic differences\textsuperscript{20} between the population studied and/or an outcome related to the prevalent genotypes in the respective parts of the world.\textsuperscript{21} So far, eight HBV genotypes (A through H) have been identified which vary in their pathogenicity and geographical distribution.\textsuperscript{21}

In conclusion, HBV DNA levels vary significantly in different states of the infection, which is helpful in diagnosing the state of infection and in turn help in designing the drug regimen. Cut-off value to classify HBeAg-negative CHB patients and inactive carriers was set at 2.5 × 10\textsuperscript{4} copies/mL for Indian population though there was enormous overlapping of HBV DNA levels. Furthermore, such studies on large samples are needed to validate this value. This value shall be imperative in the better management of this dreadful disease in our part of the world.

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References


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