

SIGNIFICANCE OF HBV DNA BY PCR OVER SEROLOGICAL MARKERS OF HBV IN ACUTE AND CHRONIC PATIENTS

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Abstract

A study was undertaken to determine Hepatitis B virus DNA (HBV DNA) by PCR in acute and chronic hepatitis B infection and to correlate it with serological markers. Three hundred and forty-five serum samples of patients from all over India were categorized into different groups according to their serological profile. HBV DNA was detected upon amplification in 166/263 patients in group A, 3/14 patients in group B, and 2/32 patients in group C, and was not detected in groups D and E. The presence of HBV DNA in 49 patients with non-replicative HBV disease, as defined by the absence of HBeAg, suggests low levels of viremia which is also supported by the abnormal liver function tests (LFTs) in these patients. In addition, HBV DNA was detected in small proportion of individuals with past HBV infection. This data suggests that, detection of HBV DNA by amplification technique serves as an important supplementary tool besides serology in a number of clinical settings, especially in determining low levels of viremia in patients with non-replicative HBV disease and chronic hepatitis, and also in a few patients with past HBV infection and who could be asymptomatic carriers of HBV infection.

Key words: *Hepatitis B virus (HBV), DNA amplification, and serological markers.*

HBV continues to be the single most cause of viral hepatitis in the developing and underdeveloped world. In addition to causing chronic liver disease and cirrhosis, it has a formidable track record of being linked to primary hepatocellular carcinoma. The diagnosis of HBV is not only imperative but also complex because of different viral antigens, which bring about varying serological profiles in different stages of the disease. The ability of HBV to induce chronic hepatic inflammation gives rise to these intricate serological profiles. Serological markers are used routinely as diagnostic and prognostic indicators of acute and chronic HBV infection. Presence of HBsAg is the most common marker of HBV infection whereas HBeAg is used as an ancillary marker to indicate active HBV infection and associated progressive liver disease. Assays for Dane particles, HBV DNA, HBeAg, and anti-HBeAg in serum are also clinically useful for assessing patients with HBV infection.

The presence of serum HBV DNA in chronic hepatitis patients indicates active virus replication. The molecular hybridization techniques like slot, or dot hybridization assays can detect <0.1 pg of HBV DNA in serum.¹ However, PCR amplification of virus DNA is potentially a more sensitive

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assay than molecular hybridization. Furthermore, DNA amplification, coupled with molecular hybridization assays, is theoretically capable of detecting attogram (10^{-18} g) levels¹ of HBV DNA in the original sample. This technique serves as a sensitive marker for the detection of HBV DNA in certain sera which are HBsAg positive even in the absence of HBeAg. Such a serological pattern corresponds to potentially infective sera and therefore the comparison of different serological markers with molecular diagnostic tests is necessary for predicting the course of chronic liver disease.

We have compared HBV DNA amplification with the various serological markers of HBV infection and assessed its positivity in acute and chronic HBV infection. We have attempted to assess low levels of viral replication in HBeAg negative states.

Patients and methods

Patients

We have analyzed 345 serum samples of patients from all over India (through Fulford representatives) for HBV DNA amplification and various serological markers. We categorized the results obtained into different groups on the basis of serological markers only (histological findings were not studied).

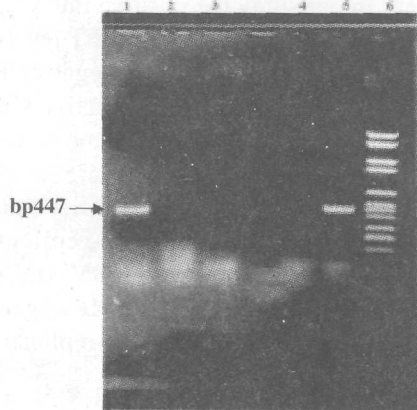
DNA Extraction

The DNA extraction was performed using QIAamp Blood kit, Qiagen^{2,3} according to the manufacturer's instructions.

Briefly, patient's sera were subjected to lysis at 70°C with 200µL lysis buffer (AL) and 20µL of protease reagent. The DNA was extracted from the lysate using absolute ethanol and subsequently purified using spin columns. Finally purified DNA was eluted from the spin columns using elution buffer (AE). The extracted DNA was subjected to amplification.

DNA Amplification

Amplification was carried out using primers obtained from the core region as reported by Mantero G *et al*⁴ oligo CH 1 – 5' TTG CCT TCT GAC TTC TTT CC 3' (1955 – 1974) 20mer, and oligo CH 2 – 5' TCT GCG AGG CGA GGG AGT TCT 3' (2401 – 2381) 21 mer (Sorin Biomedica, Italy). Amplification mixtures comprised of 20µL extracted DNA; 10pmol/µL of each oligo CH 1 and oligo CH 2; 200 µM each of deoxynucleoside triphosphates; 1U of Taq polymerase (Banglore Genei, India), 1X PCR assay buffer and sterile distilled water was added to make up the volume to 100µL. DNA amplification was carried out in Perkin Elmer 480 version Thermocycler, which comprised of initial denaturation at 94°C/3mins, followed by 30 cycles each of denaturation at 94°C/1min, primer annealing at 48°C/1min, and primer extension at 72°C/2 mins. The final extension was carried out at 72°C/7mins. The amplification products were electrophoresed on an ethidium bromide stained 2% agarose gel to visualize a 447 base pair amplified product (Figure).



- Lane 1 : Positive sample
 Lane 2&3 : Negative samples
 Lane 4&5 : Negative and Positive controls
 Lane 6 : Molecular weight marker VI
 (Boehringer Mannheim)

DNA Enzyme Immunoassay (DEIA)

The amplified products were detected by liquid hybridization technique i.e. DEIA (Sorin Biomedica) which is sensitive to

detect as few as 100pg of amplified product.⁵ DEIA was performed according to manufacturer's instructions. Streptavidin coated microtitre plates were immobilized with a 38mer single stranded, biotinylated probe (5' TCT GCT GGG GGG AAT TGA ATC TAG CTA CCT GGG TG 3')⁴ and was allowed to hybridize at 55°C for one hour with the denatured amplified products. If the patient's sera had HBV DNA then the probe would bind to the complementary sequence within the amplified product. This hybrid is further recognized by monoclonal antibodies which are specific for minor grooves of double stranded DNA. After 30 minutes of incubation at room temperature and subsequent washing, this complex was recognized by horse radish peroxidase conjugated to rabbit antimouse IgG antibody. Following the incubation at room temperature for 30 mins and washing steps, chromogen/substrate (O-phenylene-diamine hydrochloride and H₂O₂) was added and the colorimetric reaction was read at 450nm after blocking the reaction with 1N sulfuric acid.

Serological Markers

The following methods were performed for the serological markers –

1. HBsAg and anti-HBs: Abbott AUSAB EIA test,
2. Anti-HBc total and IgM: core-M IMX system Abbott labs.
3. Anti-HBeAg and HBeAg: anti-HBe2 and HBe 2 IMX system Abbott labs.

Results

The results of HBV DNA by amplification in various groups are listed in the table. Of the 345 serum samples 171 were positive by DEIA assay. All the samples were electrophoresed on 2% agarose gel and stained with ethidium bromide to visualize 447 base pair fragment. Of the 171 samples positive by DEIA 33 did not show a band on the agarose gel thereby showing the sensitivity of DEIA assay.

Discussion

Serological markers are indispensable in the diagnosis of HBV infection. HBsAg, inspite of being a common diagnostic marker of HBV infection, does not provide information about active virus replication. Also the critical issue is that the sensitivity of current serologic tests for HBsAg (1ng/mL) is lower than the minimum infectious dose of the virus (1pg/mL). On the contrary, PCR techniques with reported sensitivity in the range of 0.01 – 1 fg DNA has the potential of filling in this gap.⁶ Moreover the presence of serum HBV DNA in chronic hepatitis patients indicates

active virus replication. In group A 166/263 chronic hepatitis patients were amplification positive while 97 were amplification negative. The 263 chronic hepatitis patients were divided into 126 patients with replicative HBV disease

(as determined by the presence of HBeAg) and 137 patients with non-replicative HBV infection (defined by the absence of HBeAg), 112 of which had abnormal LFT and 25 had normal LFT levels (Table).

Table : Results of HBV DNA by PCR in different groups of patients: (n=345)

Groups	Markers	HBV DNA positive	HBV DNA negative	Total
A. Chronic hepatitis HBV	HBSAg ⁺ anti-HBcIgM ⁻ HBeAg ^{+/-}	166	97	263*
● Replicative HBV	HBeAg ⁺ , Abnormal LFT	117	9	126
● Non-replicative HBV	HBeAg ⁻ , Abnormal LFT	49	63	112
	HBeAg ⁻ , Normal LFT	0	25	25
B. Acute Hepatitis HBV	HBSAg ⁺ anti-HBcIgM ⁺	3	11	14
C. Past HBV infection	anti-HBcAg ⁺ anti-HBsAg ⁺	2	30	32
D. Hepatitis due to other causes	HBsAg ⁻ , Abnormal LFT	0	26	26
E. Healthy controls	All markers negative	0	10	10

* 263 chronic hepatitis patients were further subdivided into those with replicative HBV disease (126) and those with non-replicative HBV disease (137) of which 112 had abnormal LFT and 25 had normal LFT.

Patients positive for HBeAg or having replicative HBV disease tend to have more severe liver disease and are more infectious than those with anti-HBe. In this study 93% (117/126) chronic hepatitis patients with replicative HBV disease were positive for HBV DNA by amplification. Baker *et al*⁷ also showed a good correlation (100%) between circulating HBV DNA and replicative HBV disease. In our study 9 patients with replicative HBV disease were negative by amplification. Three of these subsequently lost their HBeAg status. In the remaining 6 patients it seems likely that some serological patterns may exist with sufficient HBeAg to permit detection but too low a titre of viral particles to permit detection of HBV DNA. We could suggest that one mode of evolution of serum HBV markers might be an initial decrease in viral particles with free HBeAg remaining in excess, followed by the replacement of HBeAg by anti-HBe.^{8,9} However in these patients we were unable to re-check their HBeAg status subsequently.

Another important issue is that seroconversion from HBeAg to anti-HBe has been ascribed to mutation in the pre C region that prohibits the synthesis and secretion of HBeAg.^{10,11} Therefore some individuals in spite of being potentially infectious remain HBeAg negative. Baker *et al*⁷ reported 78% positive rate by PCR in non-replicative HBV

disease, whereas in our study the positive rate was 44% (49/112) in patients who also had abnormal LFT levels. Thus the presence of HBV DNA in 49 HBeAg negative individuals could be due to low viremia in non-replicative HBV disease or due to the presence of a pre-core/core stop codon mutation leading to HBeAg negative states. We are aware that this needs to be further confirmed by DNA sequencing. Furthermore in 25 patients with non-replicative HBV infection and normal LFT levels, HBV DNA was not detected. Normal LFT levels, absence of HBeAg and absence of HBV DNA are suggestive of the non-replicative state in these patients.

HBV DNA was detected in 3/14 acute hepatitis patients in group B (Table) who were also HBeAg positive and had abnormal LFT. Of the remaining 11 patients who did not show the presence of HBV DNA and were HBeAg negative, 4 had abnormal LFT and 7 had normal LFT. In self-limited hepatitis due to HBV infection, HBsAg remains positive on an average for 4 weeks. It has been seen that anti-HBc IgM decline rapidly in titre after the disappearance of HBsAg in only 40% of case with self-limited acute hepatitis B; in the remainder, the decline was slow with 20% still being positive after 2 years.¹²

The presence of HBV DNA in 2/32 patients with past HBV infection in group C (Table) indicates the low replicative state of HBV even after the disappearance of HBsAg. There is a good evidence that some patients with persistent HBV infection do not have detectable HBsAg in their serum. Also a small but significant fraction of blood donors whose sera are HBsAg negative by the most sensitive tests, transmit HBV infection to recipients of their blood. Antibody to HBcAg often indicates remote HBV infection but DNA hybridization and more sensitive amplification assays have demonstrated that some HBsAg negative individuals, positive for anti-HBc, have continuing HBV replication.¹³ HBV DNA may persist for several months after clearance of HBsAg.¹⁴ Viral DNA has also been found in the serum of some individuals negative for all markers of HBV.¹⁵ Also the possibility that hepatitis B may reappear in HBsAg

negative individuals who were subsequently immunosuppressed, suggests that in some individuals the virus continues, but at a very low level in the replicative states.¹³

Serology will undoubtedly continue to be widely used in the diagnosis of HBV infection. However significant advances have been made in the diagnosis and treatment of chronic HBV infection, and the HBV DNA amplification assays serve as valuable tools to monitor all these modalities. From this study we conclude that PCR-based tests can serve as an important supplementary tool in a number of clinical settings, especially in detecting low levels of viraemia in non-replicative HBV disease and also in patients with past HBV infection. Further work up with detailed clinical and histological studies in the discordant cases are needed.

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