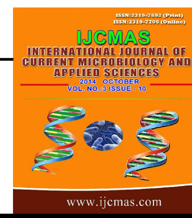
International Journal of Current Microbiology and Applied Sciences
ISSN: 2319-7706 Volume 3 Number 10 (2014) pp. 765-774
http://www.ijcmas.com



Original Research Article

HBx gene expression is a sensitive indicator of chronic Hepatitis B infection than expression of HBV Surface, Core and Polymerase gene

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A B S T R A C T

Recent studies have shown that chronic HBV infection may be responsible for development of hepatocellular carcinoma (HCC) and Cirrhosis of liver and therefore the correlation and involvement of HBx gene in the development of HBV-related HCC and Cirrhosis is one of the prime focus of researchers in the biomedical field. Many researchers are working on diagnostic front in order to fight the battle against HBV. In order to control the established infections, perfect diagnosis of chronic HBV infection is very important. Development of Molecular diagnostic programs help to introduce PCR based diagnosis as the only diagnostic method which can detect HBV DNA at low copy number ($<10^3$ copy/mL). We therefore propose to analyse the expression of all HBV genes such as pre S, Core, Polymerase and X in the infected patients from Western India. According to our analysis, HBx gene was found to be the most sensitive gene for diagnosis of chronic HBV infection as compared to other three genes. Positive expression of HBx gene was observed not only in samples below the viral load of $1x10^3$ DNA copies/mL , but also in samples with undetectable viral load when analyzed by conventional PCR for Pre S, Core and Polymerase gene. Hence we report here that HBx gene can be used as best biomarker to monitor minimal residual infection in HBV infected patients. This study further suggests the use of HBx DNA PCR as a primary approach in the diagnosis and management of chronic HBV infection.

Keywords

HBV,
HBx gene,
HCC,
Molecular diagnosis,
Infectious diseases

Introduction

Hepatitis B virus (HBV) is one of the most infectious diseases with approximately 350 million chronically infected people all around the world. Studies have shown the development of squeal diseases like liver cancer and Cirrhosis due to existence of chronic HBV infection. Prevalence and modes of transmission of HBV infection vary in different parts of the world (Maddrey, 2000). Since late 1980's and even before that, the role of HBV as the causative agent of hepatocellular carcinoma (HCC) and cirrhosis of liver had been suspected due to some positive evidences (Beasley, 1988).

The genome of HBV is partially double stranded DNA of 3.2 kilobase (kb) pairs. As it reverse transcribes from pregenomic RNA

(pgRNA) to DNA, it resembles retrovirus in this aspect (Summers and Mason, 1982). HBV genome codes for four open reading frames i.e., *S*, *C*, *P*, and *X* (Liang, 2009; Ganem and Schneider, 2001). The *S* ORF encodes for HbsAg (surface envelope protein), which is further divided into the pre-S1, pre-S2, and S regions. The *C* gene encodes HBcAg (viral nucleocapsid) when translation initiates at core or HBeAg (hepatitis B e antigen) if translation initiates at precore region. The P ORF encodes for polymerase protein which is functionally divided into terminal protein domain, reverse transcriptase domain and ribonulease domain (Liang, 2009). The X ORF is the smallest ORF which encodes the 17.5 kDa HBV protein called HBxAg (Sugata *et al.,* 1994, Guo *et al.,* 1991). HBxAg is a critical factor for viral replication, transcription regulation, influences apoptosis and cell cycle regulatory pathways (Bouchard and Schneider, 2004).

For the diagnosis and management of HBV and its sequel, there are amplified and unamplified assays along with immunohistochemical staining for HBsAg and hepatitis B core antigen (Anna *et al.,* 2001). Moreover different stages of HBV infection can be identified by serological markers like HBsAg, anti-HBs, anti-HBc by ELISA, Serum level of enzymes (SGOT, SGPT, DB, TB and ALK) by biochemical methods and HBV DNA by polymerase chain reaction and Real Time-PCR (Humaiany et al, 2009). Approximately 15% to 40% chronic HBV patients develop HCC and liver cirrhosis during their lifetime (Anna *et al.,* 2001, McMahon, 1997, Beasley, 1988). Such inactive carriers should be monitored for HBV reactivation after regular period of time. The DNA copy number is high during the initial phase of the chronic infection and HBeAg is present. However, as time progress, the carriers undergo seroconversion by clearing HBeAg and generating anti-HBe and HBV DNA level falls below the detection range of unamplified assays (below 10^5 copies/mL) (Anna *et al.,* 2001) (Hoofnagle *et al.,*1981) and (Fattovich *et al.,*1986). Unamplified assays have been reported to detected adequately early chronic infection (10^5 to 10^6 copies/ mL) (Anna *et al.,* 2001). Polymerase chain reaction is reported as the only effective means to detect HBV infection when the copy number of HBV DNA is low and HBsAg disappears from the serum of the patients (Gandhi, 2000).

The main aim was to utilize conventional PCR approach and analysis of the expression of Pre S, C, P and X gene in the infected patients having viral load ranging from undetectable to 10^{10} copies/ml and to compare their sensitivity rate for diagnosis of HBV infection. The present study focuses on the importance of HBx gene as the important biomarker for the diagnosis of chronic HBV infection even at low copy number as compared to other HBV genes.

Material and Method

Sample

The patient attending Clinic at Jaslok Hospital & Research center, Mumbai were taken for this study. Total 100 blood samples from the HBV patients were collected as per ethical approval from the ethics committee of Jaslok Hospital Research Centre (JHRC). DNA extraction from plasma was performed using Qiagen kit. HBV Real Time PCR was performed using Artus HBV TM PCR Kit. Viral copies were calculated from the Ct value using a proper standard graph wherein virus copies are expressed as DNA copies/mL. Ten normal individuals' samples were used as a negative control for HBV infection.

Concentration of DNA of HBV infection suspected samples and negative control was determined by Eppendorf Biophotometer.

When different amounts (200ng, 400ng, 600ng and 800ng) of HBV positive samples analyzed for the expression of HBx gene on agarose gel electrophoresis and visualized under UV transilluminator, 600 ng of DNA showed the highest signal intensity (Figure 1). Hence 600 ng DNA was used for each PCR reaction for all the other genes under study.

Total 100 samples were analyzed in four different groups. The groups were divided according to the viral load. The quantitative analysis of the samples was done by Real-Time PCR (ABI PRISM 7700). The first group included 25 samples ranging from 10^7-10^{10} DNA copies/mL, second group had 25 samples ranging from 10^4-10^6 DNA copies/mL, the third group included 25 samples of viral load ranging from 10^1-10^3DNA copies/mL, and the forth group comprised of undetectable viral load samples. Each group has been analyzed for the four genes of Hepatitis B virus (Pre S, Core, Polymerase and X) by Polymerase Chain Reaction (PCR)

PCR for Pre S, core, polymerase and X gene regions of HBV DNA

PCR was performed for all four genes using specific primer given in the Table 1.

HBV sequence used as reference sequence to check the primer alignment of S, C, P and X gene is HBV genotype A (GenBank: AB014370.1).

The PCR conditions for Pre S and Core gene were as: 94°C for 5 minutes, 94°C for 45 seconds, 58°C for 1 minute, 72°C for 45 seconds and 72°C for 10 minute.

PCR condition for Polymerase gene was 94°C for 5 minutes, 94°C for 30 seconds, 51°C for 30 seconds, 72°C for 1 minute, 72°C for 10 minutes.

PCR condition for X gene was 95ºC for 5 minutes, 94º C for 30 seconds, 50ºC for 1 minute, 72ºC for 1 minute (Step 2-4 37 cycles), 72ºC for 7 minutes.

The amplified DNA product from each PCR reaction for pre S,Core, Polymerase and X gene was loaded on 2% agarose gel and visualized by using U.V transilluminator and photographed.

Result and Discussion

The suspected samples for HBV infection were analyzed for the expression of Pre S, Core, Polymerase and X gene regions of HBV DNA by PCR, Real-Time PCR and DNA sequencing methods. The total 100 samples were analysed by dividing into 4 groups such as 10^7-10^{10} DNA copies/ml, 10^4-10^6 DNA copies /mL, 10^1-10^3 DNA copies/mL and undetectable viral load (Table 2). Each group comprises of 25 samples.

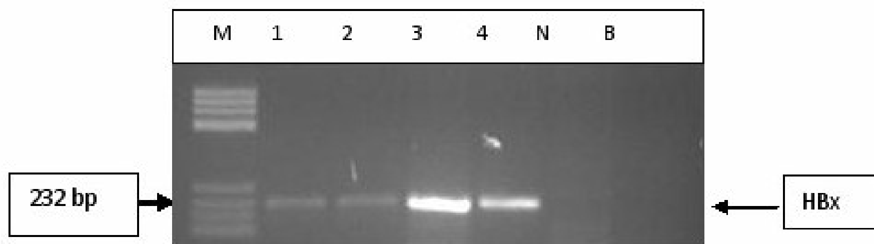
HBV infected patients with viral load 10^7-10^{10} copies/ml

Group 1 includes 25 samples with the highest viral load ranging from.10^7-10^{10} copies/ml. Figure 2 shows analysis of HBV Pre Surface, Core, Polymerase and X (HBx)gene expression at 10^7-10^{10} DNA copies/ml. It was observed that 25 out of 25 samples (100%) were positive for Pre S, 18 out of 25 samples (72%) were positive for Core gene, 23 out of 25 samples (92%) were positive for polymerase gene and 25 samples out of 25 samples (100 %) were positive for X gene indicating that Pre S and X genes were shown similar sensitivity to detect HBV infection at higher viral load.

Table.1 Forward and Reverse primers used for amplification of HBV genes by Polymerase Chain Reaction (PCR)

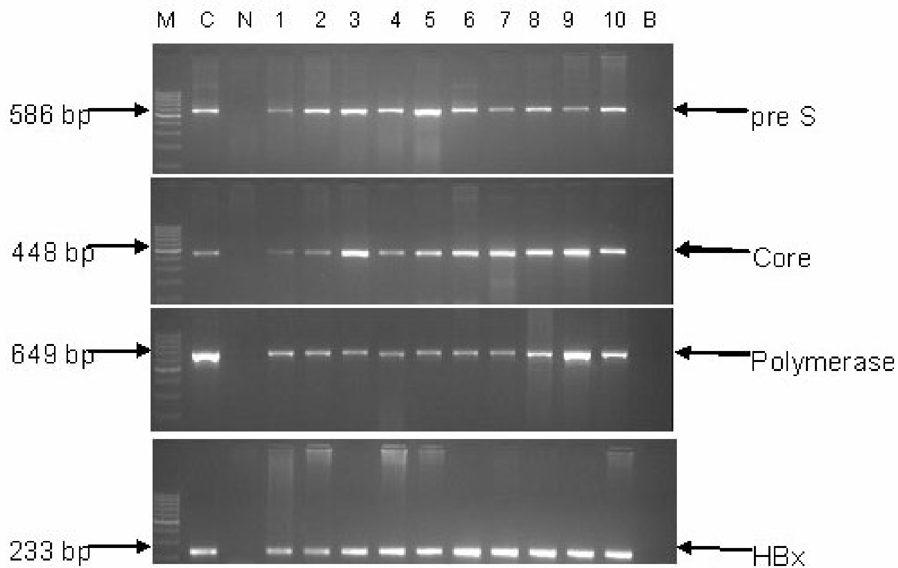
Gene	Forward Primer	Reverse Primer
pre S	5′ GGGTCACCATATTCTTGG 3′	5′ GTCCTAGGAATCCTGATG 3′
Core	5′ CTCGGATCCTTGTGACTTCTTTCCTT 3′	5′ CGGAGGCGAGGGAGTTCTTCTTCT 3′
Polymerase	5′ TGGTTATCGCTGGATGTGTC 3′	5′ CCCAAAAGACCCACAATTC 3′
X	5′TGCCAACTGGATCCTGCGCGGGACGTCCTT 3′	5’ GTTCACGGTGGTCTCCATG 3’

Figure.1 shows the gene expression of HBx gene at 200ng, 400ng, 600ng and 800ng



Key – 1- 200ng, 2- 400ng, 3-600ng and 4- 800ng of DNA, N- Normal and B- Blank

Figure.2 shows HBV gene expression of pre S, Core, and Polymerase and X gene at viral load ranging from 10^7 to 10^{10} DNA copies/mL

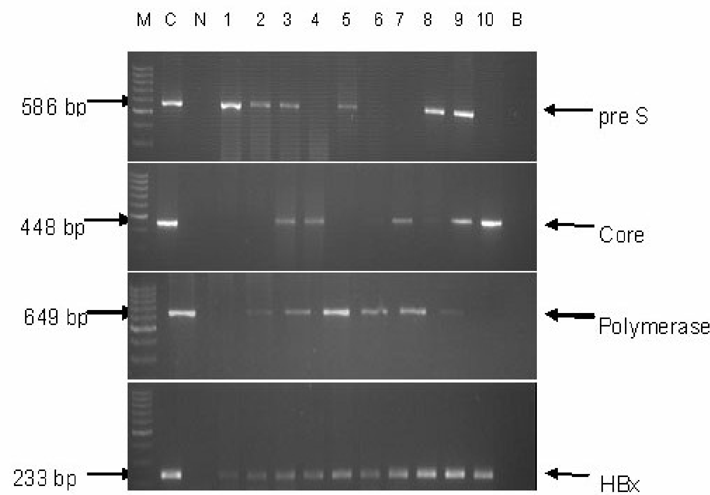


Key: M- Marker; C- Positive control; N- Normal sample; 1 to 10 – Suspected samples; B – Blank

Table.2 Expression of HBV structural genes in HBV viral load ranging from undetectable to 10^{10} DNA copies/ mL

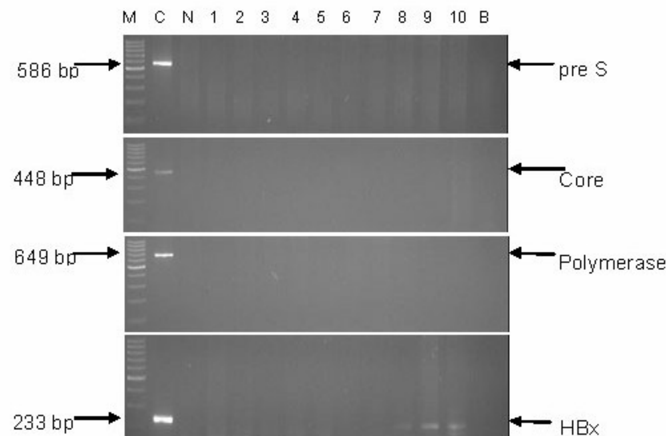
Group	Viral Load	No. of Samples	No. of positive sample			
	DNA copies/mL		pre S	Core	Polymerase	X
1	10^{7}-10^{10}	25	0	0	0	2
2	10^{4}-106	25	0	0	0	3
3	10^{1}-10^{3}	25	14	12	13	19
4	Undetectable	25	25	23	23	25

Figure.3 shows HBV gene expression of pre S, Core, and Polymerase and X gene at viral load ranging from 10^4 to 10^6 DNA copies/mL. X gene was expressed in all the samples



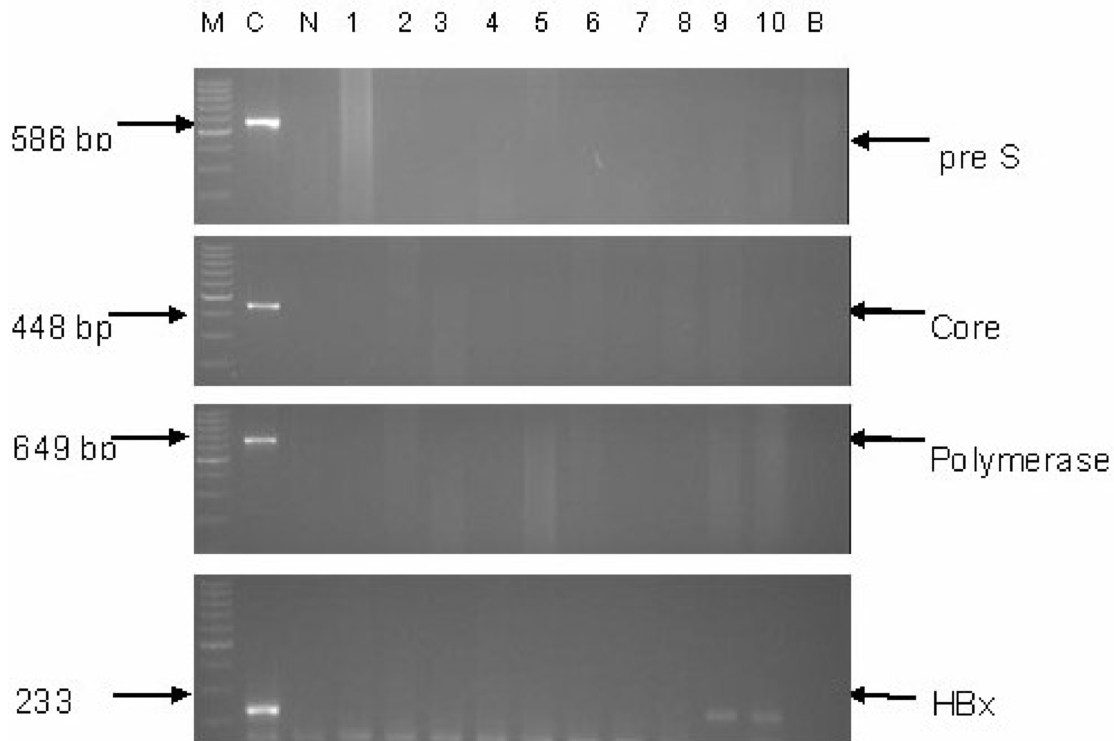
Key: M- Marker; C- Positive control; N- Normal sample; 1 to 10 – Suspected samples; B – Blank

Figure.4 shows HBV gene expression of pre S, Core, and Polymerase and X gene at viral load ranging from 10^1 to 10^3 DNA copies/mL



Key: M- Marker; C- Positive control; N- Normal sample; 1 to 10 – Suspected samples; B – Blank

Figure.5 shows HBV gene expression of pre S, Core, and Polymerase and X gene in undetectable samples



Key: M- Marker; C- Positive control; N- Normal sample; 1 to 10 – Suspected samples; B – Blank

HBV infected patients with viral load 10^4-10^6 copies/ml

Group 2 consisted of 25 samples with the viral load ranging from 10^4-10^6 copies/ml. Figure 3 indicates the expression of all four hepatitis B genes in these patients. It was observed that all four genes of Hepatitis B virus expressed in this range of viral load at the different rate of sensitivity. According to the analysis, 14 out of 25 samples (56%) were positive for Pre Surface, 12 out of 25 samples (48%) were positive for Core gene, 13 out of 25 samples (52%) were positive for polymerase gene whereas, 19 samples out of 25 samples (76 %) were positive for HBx gene indicating the higher sensitivity of HBx gene in detecting HBV viral infection.

HBV infected patients with viral load 10^1-10^3 copies/ml

Group 3 comprised of 25 samples with viral load of 10^1-10^3 copies/ml. Figure 4 shows expression of HBV Pre S, Core, Polymerase and X gene expression in these samples. It was observed that all 25 samples were negative for Pre S, Core and Polymerase gene expression. However 3 samples (12%) out of 25 samples were positive for X gene, which indicated the sensitivity of X gene amplification even at such a low viral load at which other genes did not show any expression.

HBV infected patients with undetectable viral load

Group 4 shows analysis of hepatitis B Pre Surface, Core, Polymerase and X gene expression in undetectable viral load samples (Figure 5). It was observed that there was no expression of pre S, Core and Polymerase gene in any of these analyzed samples. However, 2 patients out of 25 samples (8%) showed positive expression of X gene. We have analysed 10 normal samples for all four genes and found that all individuals were negative for HBV preS, Core, Polymerase and X gene, which confirms the absence of contamination and/or expression of these genes under normal condition. This study clearly showed that 8% of HBV infected patients having undetectable viral load amplified and expressed HBx gene and may prone to liver cancer or cirrhosis of liver in near future.

India, with more than 40 million HBV carriers is at intermediate level of HBV endemnicity (NCDC News letter 2014). Acute infection can be confirmed due to presence of serological marker called HBsAg at the level exceeding 10^5 to 10^6 copies/mL (Lok *et al.,* 2001), Serum HBV DNA and elevated level of HBeAg and transaminase (Bowden, 2002). However, people become chronic carrier because they fail to resolve acute infection. The carrier stage is marked by decrease in HBV DNA level less than 10^5 copies/mL, decline in ALT levels and declined level of anti-HBc IgM (Sablon *et al.,* 2005). The major indicators of chronic infection are persistence of HBsAg and the presence of anti-HBc in the absence of IgM anti-HBc (NCDC News letter 2014). But this diagnosis has it's own limitation because HBsAg persists for a limited time period of approximately 6 months (Gripon *et al.,* 1995). Chronic HBV infection can lead to cirrhosis and hepatocellular carcinoma and therefore need for novel therapeutic approaches are required (Schwalbe *et al.,* 2008). Some studies have suggested that HBV DNA testing has the potential of resolving concerns associated with the limitation of current diagnostic methods. In order to assess the level of viral replication and prognosis of HBV, consideration of HBV DNA assays have thought over and improved over the period of time (Grisham, 1962). The major advantage of real-time polymerase chain reaction–based assay (TaqMan) is it's lower limit of HBV DNA detection (5–10 copies/mL). Having this degree of sensitivity enables the early (before the appearance of HBsAg and anti-HBc) and chronic stage detection of HBV. Hence, nucleic acid testing (HBV DNA) has become primary approach for the diagnosis and management of HBV infection (Guidotti, 1996).

In most of the diagnostic centers the detection of hepatitis B virus infection is carried out by examining the gene amplification in the suspected samples by conventional PCR methods. Pre Surface and Core genes are most commonly preferred biomarkers to diagnose the hepatitis B infection. DNA amplification tests based on the polymerase chain reaction (PCR) Signal amplification assays have sensitivities about 1 pg of DNA, i.e.10^3-10^5 genome copies and below this level, the test showed negative result (Schwalbe *et al.,* 2008). This leads to transformation of acute infection into chronic infection if left untreated. This shows that such HBV carriers have 100-fold increase relative risk of hepatocellular carcinoma as compared to non-carriers. Many studies have reported the HBV/ HCV co-infection and hence it is recommended that viremia levels of both HBV/ HCV viruses is essential to have an effective therapeutic management of infection for the

co- infected patients (Zeinab *et al.,* 2005, Mehmet *et al.,* 2006, Semnani *et al.,* 2006). Real Time PCR is one of the sensitive technologies to detect HBV infection at undetectable level. However, because of the high cost of instrument and expensive maintenance, many Indian laboratories do not have this facility for monitoring HBV infection. Also, the cost involved for Real time PCR testing is not affordable to patients who are economically backward. Hence gene amplification and analysis by conventional PCR is an economical and pragmatic option. We therefore suggest first time to introduce HBx gene analysis by conventional PCR for diagnostics and management of chronic HBV infection.

HBx gene is the major gene of interest in our studies because out of four genes, it was observed to be the most sensitive gene for the detection of HBV infection. HBx is predominantly nuclear when expressed in cells at very low levels but becomes largely cytoplasmic as its expression level increases (Henkler *et al.,* 2001). Although these studies involved HBx expression in the absence of HBV, a dynamic distribution of HBx could be important, considering the multiple functions of HBx during the HBV life cycle, and could influence its effects on transcriptional activation in the nucleus and viral replication in the cytoplasm. HBx has been reported to be capable of binding p53, (Twu *et al.,* 1993) the TATA-binding protein (Cheong *et al.,* 1995) and a cellular protein associated with DNA repair. (Liang, 2009) These findings indicate a possible role of HBx in HBV replication (Chirillo *et al.,* 1996).

This particular study shows that approximately 8% samples with undetectable HBV DNA viral load were positive when tested for HBx gene whereas, other genes (preS, Core and polymerase) showed no expression upon amplification by PCR of the samples with undetectable viral load. Moreover, HBx gene showed progressive increase in expression with the increase in viral copy number and showed 100% expression in patients having viral load of 10^7 to 10^{10} DNA copies/mL. Over all this study first time introduces the importance of HBx gene PCR for clinical management of chronic HBV infection as it is shown to be most sensitive biomarker for HBV infection management. This approach can help clinicians from small established clinics to assess this technology for diagnosis and management chronic HBV infection to avoid further progression to HCC or liver cancer.

Acknowledgement

We acknowledge with thank the Management of Jaslok Hospital to provide the facilities to work on this project. We are thankful to Mrs. Naina Rane for her technical help during this project

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