Application of Real Time PCR for Detection and Quantification of HBV DNA in Chronic Hepatitis B Patients in Bangladesh

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Hepatitis B virus (HBV) causes a spectrum of liver diseases including acute hepatitis, chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma. The detection and quantification of hepatitis B virus (HBV) DNA plays an important role in diagnosing HBV infection as well as monitoring therapeutic responses. The present study was designed to detect and quantitate HBV DNA by Real time PCR. A cross sectional study was conducted during the period of July 2010 to June 2011. A total of 200 serologically diagnosed CHB patients were enrolled for the detection and quantification of HBV DNA by Real time PCR method at the Department of Virology, Bangabandhu Sheikh Mujib Medical University (BSMMU). Data was analyzed by SPSS. HBV DNA was detectable in 157 (78.50%) and undetectable in 43 (21.50%) patients by Real time PCR. Association of HBV DNA and HBeAg in the study population observed 74 (37%) HBeAg positive and 126 (63%) HBeAg negative patients. Among the HBeAg negative patients, viral load was less and patients were significantly older. The mean viral load of HBeAg positive and HBeAg negative was 6.40±2.042 [$\log_{10}$ (copies/ml)] and 2.83±2.55 [$\log_{10}$ (copies/ml)] respectively. HBV DNA was a more reliable indicator of the presence of virus than HBeAg, and was detected in 98.65% (73/74) HBeAg positive carriers, and in 66.67% (84/126) HBeAg negative patients. The present study observed that Real time PCR was able to detect a wide range of HBV DNA.

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Key words: Real time PCR, HBV DNA, chronic hepatitis B

Introduction

Globally, over 2 billion people are infected with Hepatitis B virus (HBV) and 370 million people are living with chronic HBV. Among them, around 660,000 die annually due to consequences of this infection.[1] Bangladesh is a densely populated country with intermediate endemicity for chronic hepatitis B (CHB) infection.[2] Studies have shown that HBV is responsible for 31.25% cases of acute hepatitis, 76.3% cases of chronic hepatitis, 61.15% cases of cirrhosis of liver and 33.3% cases of hepatocellular carcinoma in Bangladesh.[3-5] The hepatitis B surface antigen (HBsAg) positivity among the healthy adult population of Bangladesh was 7.2%-7.5%.[6-7] The natural history of chronic hepatitis B is dependent on the age of acquiring hepatitis B infection. In Bangladesh, most of the HBV infections

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occur in childhood as suggested by the high rate of interfamilial HBV infection, history of low rate of acute hepatitis and large number of younger populations' affected.[2] Serologic assays for HBV are the mainstay diagnostic tools for HBV infection. However, the advent of molecular biology–based techniques have added a new dimension to the diagnosis and treatment of patients with chronic HBV infection.[8] Viral load tests that quantify HBV in peripheral blood i.e., serum or plasma are currently the most useful and most widely used. High-sensitivity molecular assays are clearly important for the diagnosis of HBeAg negative CHB and occult HBV, where viral loads can be quite low[9]. Serum HBV DNA is the most important and reliable marker for monitoring hepatitis B viral replication[10] and HBV DNA detection and HBV DNA level measurement is essential for the diagnosis, decision to treat and subsequent monitoring of patients.[8]

The recent introduction of fluorescence resonance energy transfer (FRET)-based real-time PCR has been particularly advantageous for HBV DNA quantification because it provides high sensitivity with a much broader dynamic range than alternative assay types.[11–14] Since amplification, measurement and quantification of PCR product occur simultaneously in the same closed reaction vessel, the need for post-PCR manipulations is obviated and the risk of PCR product carry-over contamination is minimized.

Methods

This cross sectional study was carried out among chronic HBV infected patient during the period of July 2010 to June 2011. The study population consisted of 200 serologically diagnosed chronic hepatitis B patients. Collection of specimens and laboratory work was carried out at the Department of Virology, BSMMU. Samples were selected by non probability convenience sampling method. Samples were tested for HBeAg with a commercially available ELISA kit (Bio-Quant, Inc. UK) according to the manufacturer's instructions. Blood samples were collected using aseptic venipuncture technique. Approximately 5 ml venous blood was collected and was placed into a EDTA anticoagulant containing tube. The HBV DNA was quantified with a commercially available kit (RoboGene HBV DNA Quantification Kit, Lot no- 009, Germany) according to the manufacturer's instructions. Results were expressed as mean ± standard deviation (SD) or percentage. Statistical analysis of HBV-DNA value was performed after $\log_{10}$ conversion. Fisher's Exact test was used for comparison. Pearson Chi-square test was performed for correlation analysis. Statistical analysis was made using SPSS 17.0 software, and *p*-value of <0.05 considered as significant.

Results

In the present study, out of the total 200 CHB patients tested by Real time PCR, 157 (78.50%) had detectable HBV DNA. Of them, 123 (61.50%) were males and 34 (17.00%) were females. The mean viral load of these patients was 5.36±2.24 [$\log_{10}$ (copies/ml)] and 5.07±2.17 [$\log_{10}$ (copies/ml)] respectively. On the other hand, HBV DNA was undetectable in 43 (21.50%) patients by Real time PCR. Among these patients, 35 (17.50%) were males and 08 (4.00%) were females (Table I).

Detection of HBV DNA by Real time PCR in CHB patients according to HBeAg status is shown in Table II. Among the total study population, 74 (37%) were HBeAg positive CHB patients. Of these, Real time PCR detected HBV DNA in 73 (98.65%), where 56 (76.71%) were males and 17 (23.29%) were females; their mean viral load was 6.54±1.77 [$\log_{10}$ (copies/ml)] and 6.36±2.36 [$\log_{10}$ (copies/ml)] respectively. Only 1 (1.35%) HBeAg positive male patient had undetectable HBV DNA. On the other hand, among the total study population, 126 (63%)

were HBeAg negative CHB patients. Of these, 84 (66.67%) had detectable DNA by Real time PCR, where 69 (82.14%) were males and 15 (17.86%) were females; their mean viral load was 4.34±1.99 [log_{10} (copies/ml)] and 3.82±1.59 [log_{10} (copies/ml)] respectively. Of the HBeAg negative patients, 42 (33.33%) had undetectable DNA by Real time PCR, where 32 (76.20%) were male and 10 (23.80%) were female patients. However, no significant difference was observed in the mean viral load among male and female patients.

The association between ALT and HBV DNA levels among the CHB patients is shown in Table III. Out of the total study population, 157 (78.5%) had detectable HBV DNA by Real time PCR. Among them, 64 (40.76%) had normal and 93 (59.24%) had increased ALT levels. However, among the 43 (21.5%) patients who had undetectable HBV DNA, 29 (67.44%) had normal and 14 (32.56%) had increased ALT levels ($p<0.05$).

Table I: Detection of HBV DNA by Real time PCR (n= 200)

Detectable HBV DNA			Undetectable HBV DNA		
Total N %	Male N %	Female N %	Total N %	Male N %	Female N %
157(78.50) 5.29±2.21*	123(61.50) 5.36±2.24*	34 (17.00) 5.07±2.17*	43(21.50)	35(17.50)	08(4.00)

* The mean viral load [log_{10} (copies/ml)]

Table II: Detection of HBV DNA by Real time PCR in CHB patient according to HBeAg status

HBeAg status	Detectable HBV DNA						Undetectable HBV DNA		
	Male	Viral load	Female	Viral load	P value	Total (%)	Male	Female	Total (%)
Positive n = 74	56 (76.71)	6.54±1.77#	17 (23.29)	6.36±2.36#	0.234*	73 (98.65)	1 (1.35)	0	1 (1.35)
Negative n = 126	69 (82.14)	4.34±1.99#	15 (17.86)	3.82±1.59#	0.242*	84 (66.67)	32 (76.20)	10 (23.80)	42 (33.33)
Total n = 200	125 (62.50)		32 (16.00)			157 (78.5)	33 (16.50)	10 (05.00)	43 (21.5)

#HBV DNA (Mean±SD) [log_{10} (copies/ml)].
Figure within parentheses indicates percentage.
*Pearson Chi- square test was done.
P<0.05 indicates significant.

Table III: Association of HBV DNA with ALT in CHB patients

HBV DNA (by Real time PCR)	Total cases	ALT (U/L)		p
		Normal N %	Increased N %	
Positive	157	64 (40.76)	93 (59.24)	0.003
Negative	43	29 (67.44)	14 (32.56)	
Total	200	93	107	

Discussion

Hepatitis B virus causes chronic infection in approximately 400 million people in the world. Most carriers of chronic HBV, including Asians, Africans, and a proportion of individuals from Mediterranean countries acquire the infection at birth or within the first 1 to 2 years after birth.[15] In the present study, 200 serologically diagnosed CHB patients were enrolled for the detection and quantitation of HBV DNA by Real time PCR method. In the study, among the total study population, 157 (78.50%) had detectable and 43 (21.50%) had undetectable HBV DNA by Real time PCR (Table I).

The mean viral load of HBeAg positive and HBeAg negative patients in the present study was 6.40±2.04 [$\log_{10}$ (copies/ml)] and 2.83±2.55 [$\log_{10}$ (copies/ml)] respectively. Previous studies show that in comparison to HBeAg positive CHB patients, HBeAg negative patients have lower serum HBV DNA and have more advanced disease as evidenced by liver histology.[16] Similar result was also observed in the present study. A study from Iran showed that HBV DNA levels were higher in HBeAg-positive patients, where 87% patients were negative for HBeAg.[17] In a study from Korea, it was observed that the median serum HBV DNA for HBeAg negative patients was approximately two logs lower than HBeAg positive patient, regardless of ALT level.[18] Another study from Bangladesh showed high DNA load in 96% HBeAg positive patients compared to only 54.1% among HBeAg negative patients.[3] Chi et al.[19] also observed that mean serum HBV DNA levels were significantly lower in HBeAg-negative patients. In the study, HBV DNA levels were also significantly lower in HBeAg negative patients (Table II). Another study showed the viral load in HBeAg-positive patients was higher than in HBeAg-negative individuals.[20]

Among the 200 cases, all HBeAg positive cases were also positive for HBV DNA by Real time PCR except 1 case, which had undetectable HBV DNA. This case was tested for HBV DNA by Real time PCR again but DNA was undetectable again and repeat test of HBeAg also showed positive result. However, the HBeAg status was not re-checked subsequently in this patient. In this case, DNA was probably undetectable due to antiviral therapy. During treatment, HBV DNA becomes undetectable before seroconversion of HBeAg. A study from India reported three cases which were HBeAg positive but were negative for HBV DNA by PCR.[21] In another study, 9 patients with replicative HBV disease were negative by amplification. Three of these patients 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. It has been suggest that one mode of evolution of serum HBV markers may be an initial decrease in viral particles with free HBeAg remaining in excess, followed by the replacement of HBeAg by anti-HBe.[22] Among the HBeAg negative patients in the present study, 84 (66.67%) had detectable while 42 (33.33%) had undetectable HBV DNA by Real time PCR (Table III). In HBeAg-negative patients, detection of HBV DNA is the only reliable marker of active HBV replication. Among the HBeAg positive cases the mean viral load of males and females were 6.54±1.77 [log_{10} (copies/ml)] and 6.36±2.36 [log_{10} (copies/ml)] respectively. On the other hand, among the HBeAg negative patients the mean viral load of males and females were 4.34±1.99 [log_{10} (copies/ml)] and 3.82±1.59 [log_{10} (copies/ml)] respectively. However, no significant differences in viral load were observed among the genders. Studies from Nigeria and America also found similar result.[23-24]

In the present study, the ALT levels in patients with detectable HBV DNA were significantly higher than those who were HBV DNA undetectable carriers (Table III). Zhang et al.[25] also found a significant relationship with HBV DNA and ALT levels. A study from BIRDEM, Bangladesh, also observed a good association between positive HBV DNA and raised alanine transaminase.[26] In low-income countries like Bangladesh, many patients refuse to do PCR and / or liver biopsy procedures as they cannot afford to bear the cost of these tests. Therefore, elevated alanine aminotransferase (ALT) levels can be used as indicators of liver cell injury and as non-invasive diagnostic tests in routine laboratory tests for these patients.

Conclusion

The present study observed that, Real time PCR is able to detect a wide range of HBV DNA. Patients who were HBeAg negative had lower viral load and were significantly older. ALT level was raised in patients with detectable HBV DNA. However, this study was limited by lack of genotyping for HBV and determining the frequency of precore/core promoter mutation among HBeAg negative CHB patients. This aspects need to be evaluated further in future studies with large number of CHB patients.

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