

Full Length Research Paper

Detection of diagnosis escape variants of Hepatitis B virus by in house polymerase chain reaction assay

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Quantitative detection of Hepatitis B virus (HBV) DNA in serum by real time polymerase chain reaction (PCR) assay emerged as a gold standard in guided anti viral therapy. HBV, due to its quasispecies nature, whether evade detection by the commercially used diagnostic PCR assays have not been elucidated so far. In this study, an in house nested PCR assay employing mismatched primer was found to detect HBV variants that escape detection by a commercial real time (Cobas TaqMan HBV test) PCR assay. Sera of 178 HBsAg +ve subjects were screened for HBV DNA by both the assays and sequence verified. The present assay detected 80% of 108 real time PCR positive subjects of which, 79.7% had single band (858bp) of HBV DNA and 20.3% showed mixed type (858 + 192 bp). HBV specific 192 bp amplicon containing A1762T and G1764A mutations has also been detected in 5 (7.1%) out of 70 real time PCR negative subjects. The data provided important information on persistent heterogeneity of HBV in single infected individual that escape routine detection by the commercial assay, and, thus, might have implication on the estimation of viral load as well as treatment outcome in HBV infection.

Key words: Hepatitis B virus, in house polymerase chain reaction (PCR), real time polymerase chain reaction (PCR), diagnosis escape variants, viral load.

INTRODUCTION

Hepatitis B virus (HBV), due to lack of proofreading ability of its polymerases, can exist as closely related yet genetically distinguished variants known as quasispecies. In this settings, the variants possessing highest environmental fitness may exist as predominant types while others remain as fluctuating proportions of minor variants (Blum, 1993). This quasispecies nature has further been attributed as one of the possible factor responsible for immune escape and drug resistance phenomena (Martin et al., 2006). HBV variants having mutations in the pre-core region and unable to synthesize e antigen (HBeAg) has been shown to emerge during anti-HBe seroconversion and interferon therapy (Günther et al., 1992; Okamoto et al., 1990). The ability of these variants to replace wild-type HBV after anti-HBe seroconversion has also been reported in carriers

(Okamoto et al., 1990; Hamasaki et al., 1994).

HBV core deletion variants detected by nested PCR assay has been found to be associated with advanced cirrhosis of liver (Baginski et al., 1996). In Europe and Asia, about 50 to 80% of chronic HBeAg negative patients harbor precore and core promoter variants of HBV (Funk et al., 2002). The prevalence of precore mutants in India are reported to be 13.6% in acute and 12.5% in fulminant HBV infection (Gandhe et al., 2003). Up to 50 to 80% of patients with HBeAg negative chronic hepatitis in Europe and Asia possess precore and core promoter variants of HBV (Funk et al., 2002) while precore mutants are found to be associated with 13.6 and 12.5% of acute and fulminant cases of HBV infection respectively from India (Gandhe et al., 2003). In the era with the advent of improved molecular diagnostic methods, the real time polymerase chain reaction (PCR) assay for detection of viral nucleic acids emerged as the gold standard for quantitative detection of viral nucleic acids and monitoring viral loads during anti viral therapy in HBV infection, in treatment of HBV infection. At

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present, it quantitative detection of serum HBV DNA as viral load by such procedures is being used to assess severity of liver disease, decision to receive antiviral therapy as well as treatment response and estimation of infectivity of a patient given individual (Gish and Locarnini, 2006). The commercially available real time HBV DNA PCR assays, employ primers and probes targeting the pre-core/core region of the HBV genome to calculate the viral load of respective patients. Despite the reported quasispecies nature of HBV, detection of the persistent heterogeneous viral population in single patient by targeting extended pre-core/core region of HBV genome has not been elucidated in routine clinical registry. Whether such variants can escape detection by the commercial real time PCR assay and thus not been reflected in estimation calculation of the viral load has not been evaluated and is yet to be ascertained. Based on previous information's, Hence, attempt has been made in the present study to evaluate an in-house nested PCR assay targeting the whole preC/C regions and part of the X gene of HBV. In this assay, the inner reverse primer is mismatched to the complementary consensus sequence of HBV genome for detection identification of variant HBV genomic fragments not detected by a commercially available (Cobas[®] TaqMan[®], Roche Diagnostics, USA) real time PCR assay, possibly coexistent majority and/or minority genomic fragments which remain undetectable by a commercially available (Cobas[®] TaqMan[®], Roche Diagnostics, USA) real time PCR assay.

One of the inner primer mismatched to the consensus sequence of the targeted HBV region has been employed in the assay for identification of possibly coexistent majority and/or minority genomic fragments which remain undetectable by a commercially available (Cobas[®] TaqMan[®], Roche Diagnostics, USA) real time PCR assay.

MATERIALS AND METHODS

Patients and clinical samples

Sera obtained from 178 treatment naïve and HBsAg positive patients who attended the Asian Institute of Gastroenterology, Hyderabad, India during the period of March 2010 to September 2010 served as the study material. One hundred and seventy eight serum samples obtained from different treatment naïve donor subjects who were HBsAg positive and attended the Asian Institute of Gastroenterology, Hyderabad, India during the period of March 2010 to September 2010 were included in the study. Serum samples were analysed for ALT, HBeAg and anti HBe status by routine clinical biochemistry and ELISA (Amar-EASE, Taiwan) procedures as per manufacturer's instructions. All the subjects were analysed for their ALT, HBeAg and anti HBe status by clinical biochemistry procedures and ELISA (Amar-EASE, Taiwan) respectively as per manufacturer's instructions. Same samples were subjected to real-time and in house PCR assay upon extraction of viral nucleic acid. All sera were negative for anti-HCV antibody upon testing with commercial enzyme immunoassays (Biokit, Spain). Informed consents were taken from the patients subjects and the study was approved by the institutional review

board.

Extraction of HBV DNA

Viral DNA extracted from sera by the High Pure System Viral Nucleic Acid Kit (Roche Molecular Systems Inc, USA) as per manufacturer's protocol. Extracted HBV DNA is used as template for both the real time and in house PCR assay.

Real time PCR assay

Amplification and quantitation of extracted HBV DNA was performed by Cobas[®] TaqMan[®] 48 Analyzer (Roche Diagnostics, USA) using real time Cobas[®] TaqMan[®] HBV test kit (Roche Molecular System, USA) as per manufacturer's instructions, which rely on PCR amplification primers that define a sequence within the highly conserved pre-core/core region of the HBV genome using Cobas[®] TaqMan[®] 48 Analyzer (Roche Diagnostics, USA) as per manufacturer's instructions.

In-house nested PCR assay

1st PCR

Extracted DNA (10 µl) were amplified in a 50 µl reaction volume containing primers (Maruyama et al., 1998) S1(nt 1653-1672,5'-cataa/gagga/ctctt/ggact-3') and A1(nt 2487-2511,5'-caggt/acagt/agaag/ataaa/agccc-3') 5 µl (40 pmole/µl) each, 5 µl 10x reaction buffer containing MgCl₂ (25 mM), 1.0 µl dNTPs (10 mM), 0.5 µl Taq polymerase (3 u/ml) and 32.5 µl sterile dH₂O. The reaction was performed in a programmable thermocycler (Eppendorf, Germany) using following protocol: initial denaturation at 94°C for 3 min followed by 30 PCR cycles each of which involve denaturation at 94°C for 15 s, annealing at 55°C for 30 s and extension at 72°C for 45 s respectively. After completion of 30 cycles, a final extension step was performed at 72°C for 5 min to obtain a 858 bp amplicon. The last three steps were repeated 30 times and were followed by the last step – 5 min at 72°C to obtain a fragment of 858 bp amplicon.

2nd PCR

The 1st PCR product amplicon (5 µl) served as template for the 2nd PCR reaction using inner primer sets (Maruyama et al., 1998) S2 (nt 1744-1761,5'-gggag/gagat/taggt/taa-3') and A2 (nt 1917-1936,5'-agaag/ctcca/aattgc/tttat-3') to obtain the desired product of 192 bp. The A2 primer is mismatch to the consensus/wild type sequence of the 858 bp amplicon generated by the 1st PCR. All other conditions of the reaction were identical to the 1st PCR reaction except the duration of the final extension step at 72°C was reduced to 3 min to obtain a product of 192 bp. Negative controls were included in each reaction to rule out any contamination from previously generated amplicons. Possibility of cross over and carry over contaminations were taken care of by employing appropriate measures with simultaneous processing of respective negative controls. A 10 µl aliquot from each amplified sample along with a 100 bp reference DNA ladder was subjected to 2% agarose gel electrophoresis for 45 min at 100 volts. Upon staining with ethidium bromide, respective DNA bands were visualized on the gel through an UV transilluminator (Vilber Lourmat, France). Each amplified sample (10 µl) was subjected to 2% agarose gel electrophoresis (100 V, 45 min) separation along with a 100 bp DNA ladder and visualized by UV fluorescence after staining with ethidium bromide. Densitometric estimation of the DNA bands was performed by

Table 1. Demographic, biochemical and virological features of real time PCR positive subjects.

Characteristics	All subjects (n = 108)
Age (years); Mean \pm SD (Range)	40.41 \pm 14.13 (13 – 68)
Sex (% male)	87.96
SerumALT (IU/ml); Mean \pm SD (Range)	98 \pm 217 (15 – 1880)
Viral load by real time PCR-Log (Mean \pm SD) Copies/ml (Range) ¹	4.69 \pm 2.14 (1.54 – 8.81)
No. positive by in house PCR assay	88 (81.4%)

Bio-Capt gel imager system (Vilber Lourmat, France) using the software Image J 1.42 (Broken Symmetry Software, USA).

Semiquantification of the DNA bands was performed by densitometry upon capturing the gel image by Bio-Capt (Vilber Lourmat, France) using the software Image J 1.42 (Broken Symmetry Software, USA).

Direct sequencing

Direct sequencing of in house PCR generated genomic fragments fractionated PCR fragments was performed employing inner sense and antisense oligonucleotide primers. As mentioned previously, through Ampli-Taq facilitated cycle sequencing reaction by the dyedeoxy terminator method (Applied Biosystems, USA) was employed using 373A automated DNA sequencer (Applied Biosystems, USA) as per manufacturer's instructions.

Statistical analysis

Descriptive statistics (mean, standard deviations), Student's *t*-test and Fisher's exact tests were performed as and where applicable using SPSS software, version 13.0 (SPSS, Inc., Chicago, IL). A value of *P* < 0.05 was considered statistically significant.

RESULTS

Application performance of in house PCR on real time PCR positive subjects

Out of 178 HBsAg positive patients subjects tested, 108 were appeared positive by real time PCR assay of which, majority were male with raised ALT values (Table 1). About 80% were negative for HBeAg and as well as positive for anti HBe indicating seroconversion. Among the 108 real time PCR positive subjects, the in house PCR assay detected HBV genomic fragments in 88 subjects. About 61% showed positivity by the presence of 858 bp amplicon after completion of 1st PCR and nearly 20% in addition were positive by the presence of 192 bp band after completion of the 2nd PCR, it was detected positive after completion of 1st PCR by the presence of 858 bp amplicon while nearly 20% in addition were positive upon completion of the 2nd PCR having an amplicon of 192 bp specific to the inner primers used along with the 858 bp amplicon derived from the 1st PCR. Since the inner primers A2 has no complementary sequence in the wild type 858 bp amplicon, no 2nd PCR product was evident in those subjects having only the

consensus/wild type sequence in the 1st PCR derived 858 bp product. On the otherhand, subjects having altered sequences in the 858 bp product but complementary to the reverse primer A2 facilitated effective binding of the primer thus yielded the desired 192 bp amplicon after the completion of the 2nd PCR. The inner primers had no role in amplification of the 1st PCR product since appearance of this 858bp band after 2nd PCR was found to occur through extra cycling conditions as confirmed by performing the 2nd PCR without any primers. Patients subjects having more or less identical viral loads as estimated by real time PCR differ qualitatively in terms of having single or dual amplicons (Figure 1). Table 2 depicted the comparative features of patients subjects having single (858 bp) and mixed type (both 858 and 192 bp) of HBV genomic fragments where no significant differences of viral load and ALT values were seen between the groups. Considering the real time assay as the gold standard, the in house assay appeared less sensitive but was found to meet the acceptable standards of a diagnostic assay (Table 3).

Application performance of in house PCR on real time PCR negative subjects

Out of 70 real time PCR negative serum samples where target has not been detected by the real time PCR, and no viral load can be measured, the in house assay detected 192 bp DNA band in 5(7.14%) of them indicating positivity for HBV DNA (Figure 2). identified 5(7.14%) subjects as positive for HBV DNA as evident by the presence of 192 bp DNA band (Figure 2). by detecting the single 192 bp genomic fragment of specific HBV origin (Figure 2). Direct sequencing of 192 bp product obtained from these subjects revealed dual mutations (A1762T and G1764A) at the basal core promoter (BCP) region of the HBV genome.

DISCUSSION

Diagnostic PCR assays used to detect HBV DNA rely on targeting and/or amplifying a specific region of the HBV genome as the representative of the existing viral population in infected individual. In spite of known quasispecies nature of HBV as a source of genomic

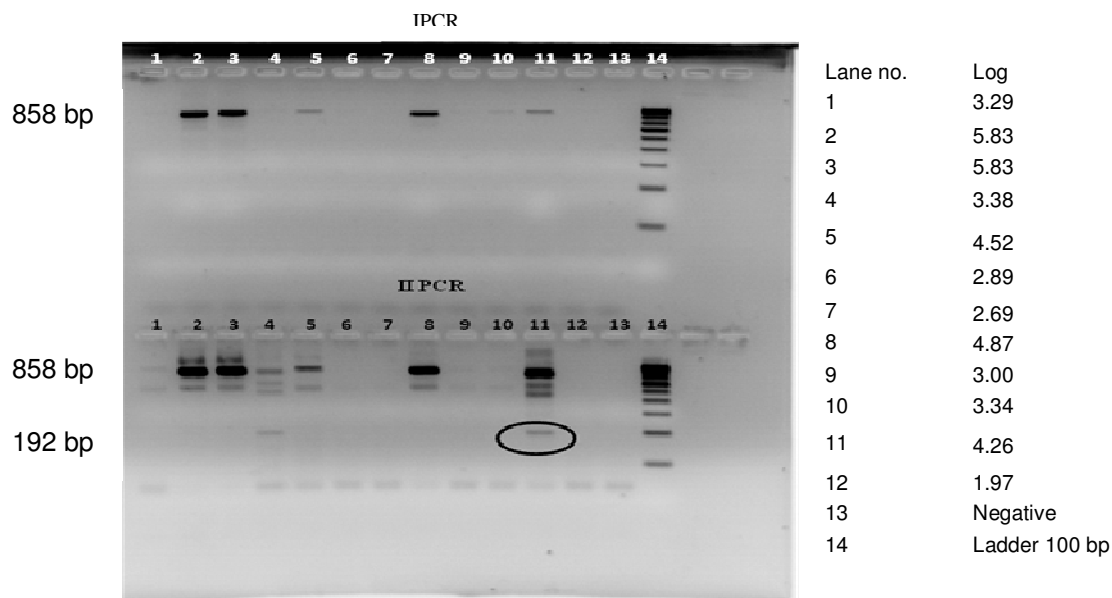


Figure 1. Population heterogeneity of HBV detected by in house PCR assay with corresponding viral load estimated by real time PCR.

Table 2. Baseline features of In-House PCR positive subjects harbouring single and mixed type of HBV genomic fragments.

Parameters	Groups	
	Single type (858 bp only)	Mixed type (858 + 192 bp)
No. (%)	66 (61)	22 (20.3)
Age (yrs) Mean \pm SD (Range)	39.4 \pm 13.9 (13-67)	45.2 \pm 12.0
Male (%)	87.8	95.4
ALT (IU/ml) Mean \pm SD (Range)	112.42 \pm 253.14 (15-1880)	83.7 \pm 85.6 (26-420)
Viral load by real time PCR-Log (Mean \pm SD) Copies/ml (Range)	5.32 \pm 2.0 (2.80-8.81)	5.02 \pm 1.84 (2.89-8.81) *

*p < 0.0001

variants, attempts has not been made to detect such variants in routine clinical diagnosis. By strategic employment of a mismatched primer, the present assay was able to detect both wild type and variant HBV genomic fragments in a single HBV patient. In light of the importance of HBV quasispecies as the source of HBV variants, the present assay detected two HBV genomic fragments simultaneously in a single HBV infected subject showing HBV genome heterogeneity at least in a group of infected subjects. Furthermore, the assay also detected HBV DNA in at least 7% of the real time PCR (Cobas[®] TaqMan[®], Roche Diagnostics, USA) negative patients subjects, thus, can be termed as diagnosis escape variants (DEV) of HBV. The absence of HBV specific amplicon in serum of other patients strongly argues against the theoretical possibility of a PCR artifact.

Furthermore, direct sequencing of the amplicon confirmed the presence of region specific fragments of HBV genome thus opposing the possibility of any non specific integration. Moreover, the present assay was

able to detect two different genomic fragments (858 and 192 bp) simultaneously as possible majority and/or, minority genome populations of HBV in single individual by extending the target HBV region used in the real time (Cobas[®] TaqMan[®] HBV) assay. Thus, in HBV infection, the possible outcomes indicated by the present assay are patients subjects having only 858 bp bands as representative of majority genomes, subjects with only 192 bp bands representing the minority genomes and subjects having both the fragments representing coexistence of majority and minority genomes of HBV. Among the four open reading frames of the HBV genome that is, S (surface), C (core), P (polymerase) and X (HBx), the X gene is least studied since it is considered less important for viral assembly proteins. Interestingly, HBx has been shown to stimulate HBV promoter and enhancer activity (Nakatake et al., 1993; Spandau and Lee, 1988). Importance of the HBx gene expression during HBV life cycle has also been implicated (Chen et al., 1993; Zoulim et al., 1994), the ability of HBx to

Table 3. Diagnostic potential of the In house PCR assay.

Parameters	Percentage	Range (%) at 95% CI
Sensitivity	80.56	71.82-87.54
Specificity	100	54.05-100
Disease prevalence	94.74	88.89-98.03
Positive predictive value	100	95.81-100
Negative predictive value	22.22	8.68-42.27
Efficiency*	94.74	
Z-Index #	94	

* The proportion of test results that are correct, # Overall measure of reliability of a diagnostic test.

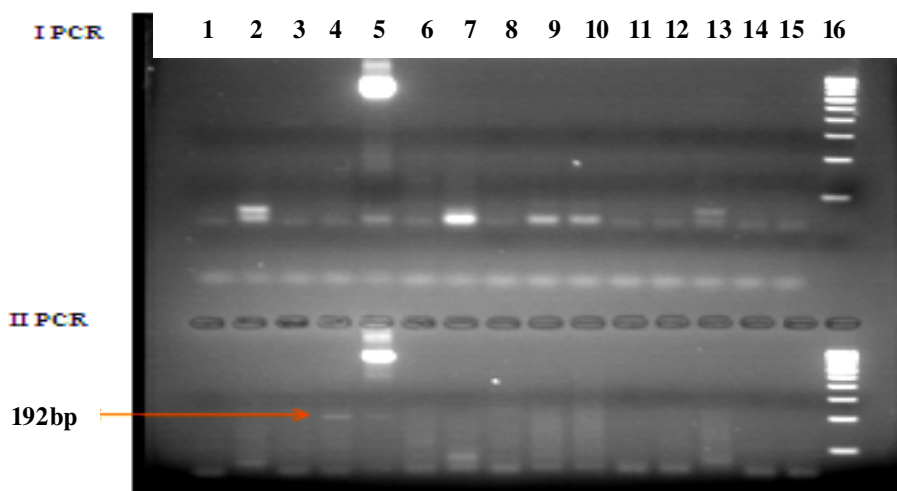


Figure 2. Detection of HBV DNA by in house PCR assay in real time PCR negative subjects (Lane 4 = 192 bp HBV fragment; Lane 5 = known +ve control showing 858 HBV fragment; Lane 16 = 100 bp DNA Ladder).

stimulate HBV promoter and enhancer (Nakatake et al., 1993; Spandau and Lee, 1988) as well as the necessity for HBx gene expression during the viral life cycle (Chen et al., 1993; Zoulim et al., 1994) has been reported. Considering the importance of the X region, the present assay targeted the precore/core and part of the X gene including the EnII/BCP region in contrast to the commercial PCR assay which rely on targeting the precore/core region only. In the present assay, part of the X gene including the EnII/BCP region along with the whole of precore/core region of the HBV genome has been amplified in contrast to the amplification of only precore/core region by the commercial real time PCR assay. The primers used were previously described to detect pre-core stop codon mutations (Maruyama et al., 1998) where generation of 858 bp but not the 192 bp fragment is mentioned. Since the 192 bp fragment is detectable in about 20% of our subjects, it might happened that this particular fragment has not been detected in earlier study (Maruyama et al., 1998) because of the small number ($n = 35$) of their study subjects. At present, it is not known whether the

appearance of these variants in some HBV infected patients subjects is specific to the geographic locations region or HBV genotypes, although genotype D is predominant in this part of the country (Mukherjee et al., 2009). The purpose of the present study was purely diagnostic rather than identification of HBV mutants, disease severity or treatment outcomes. The basis of appearance of the 192 bp fragment in some patients, infected subjects but not in others can be explained by the use of the inner reverse primer (A2), which is mismatch to the consensus sequence of the 858 bp region. Patients harboring genomic variants and having altered sequence in the primer binding site but compatible to the primer A2 were thus detected positive by visualization of desired 192 bp amplicon. It is possible that some infected subjects might harbor two versions of the said 858 bp fragments. One fragment having complete consensus/wild type sequence remain inaccessible to the inner reverse primer (A2), thus appeared as single band of 858 bp even after completion of the 2nd PCR while the other one had altered sequence in the primer binding site and appeared accessible to the inner reverse

primer (A2) and successfully amplified to generate the desired 192 bp amplicon. However, the possible role of core deletions altering the primer binding sites has not been clarified in this preliminary study which needs phylogenetic profiling using extended whole HBV genome amplification and sequencing of HBV genome in larger cohorts of defined disease categories. Although measurement of serum HBV DNA levels is an essential tool to assess disease severity and monitoring of anti viral treatment response, patients having low serum HBV DNA levels bearing risk of progression to cirrhosis and hepatocellular carcinoma (HCC) has been reported (Shao et al., 2007).

However, despite the notion that monitoring changes in serum HBV DNA levels is an essential tool, its fluctuating nature has been reported where patients having low HBV-DNA levels have a risk of progression to cirrhosis and hepatocellular carcinoma (HCC) (Shao et al., 2007). In patients with HBV-related decompensated cirrhosis, no association was found between serum HBV DNA and respective histological activity in the liver (Kim et al., 2010). Insufficiency of serum HBV DNA level alone to begin anti viral treatment has also been reported (Bárcena and García, 2009). Furthermore, non association of serum HBV DNA with the histologic activity in patients with HBV-related decompensated cirrhosis and insufficiency of serum HBV DNA level alone to begin treatment has also been reported (Kim et al., 2010; Bárcena and García, 2009). In this regard, existence of HBV heterogeneity in some infected subjects as indicated by the present assay might have implications on their estimated levels of serum HBV DNA viral load. As evident by the present assay, HBV patients having identical viral loads as estimated by the commercial PCR assay may differ virologically in respect to the prevailing homogeneous or heterogenous genomic population of HBV. In other way, HBV infected subjects having identical viral loads as estimated by the commercial PCR assay may differ virologically in respect to the prevailing homogeneous or heterogenous nature of the HBV genome.

Conclusion

In conclusion, the present assay is the first of its kind to detect diagnosis escape variant (DEV) of HBV that escape detection by a highly sensitive commercial real time PCR assay. Appropriate customization of the present assay with increased sensitivity can pave the way for its use as an diagnostic add-on to the current practice of detection and quantification of HBV in patients diagnostic armatorium aiming individualized treatment for better patient management in HBV infection.

In conclusion the present assay simultaneously detected two genomic fragments of HBV in a single patient and is the first of its kind to detect diagnosis

escape variant (DEV) of HBV that escape detection by a highly sensitive commercial real time PCR assay in use. Customization of the present assay with increased sensitivity might help as an add-on to the current diagnostic armatorium aiming individualized treatment for better patient management in HBV infection.

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