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A Multiplex Snapshot Assay for Rapid and Simultaneous Detection of Major HBV Drug Resistance Mutations Along with Precore (Pre-C) and Basal Core Promoter (BCP) Mutations.

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ABSTRACT

Antiviral drug resistance mutations at L180M and M204V/I in HBV polymerase gene are critical in determining the success of long-term therapy for chronic hepatitis B. Detection of BCP mutations at A1762T/G1764A along with G1896A in pre-C variant, is associated with lower rates of interferon response. A multiplex SNapShot assay for simultaneous detection of these five mutation has been developed. Assay involves multiplex PCR in a single tube reaction followed by minisequencing (SNaPshot) technique (multiplex primer extension and capillary electrophoresis). The accuracy of detection of the mixed infections was same by SNaPshot method compared with that achieved with the DNA sequencing methods. The results of the present study indicated that the SNaPshot technique accurately detects five major HBV mutations and is able to be readily applied as an important tool in HBV treatment and monitoring.The assay described here offers a rapid, sensitive, cost-effective and specific approach to the analysis of HBV mutations having high clinical significance.

Keywords: SNapShot, Hepatitis B Virus, multiplex PCR, Basal core promoter mutations

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INTRODUCTION

The hepatitis B virus (HBV) infection is major cause of hepatitis as well as aggressive and advanced liver disease, including cirrhosis and hepatocellular carcinoma in human patients(1). Nucleos(t)ide analogue reverse transcriptase inhibitors (NRTIs) are an essential tool for the treatment of hepatitis B virus (HBV)-associated liver disease because of their effective antiviral activity in the absence of significant side effects and major contraindications(2). Treatment of chronic hepatitis B (CHB) is geared to achieving continuous suppression of hepatitis B virus (HBV) replication and reduction of liver disease, with the aim of averting liver failure, cirrhosis, and hepatocellular carcinoma(3). Antiviral drug resistance in HBV is associated with mutations in the highly conserved YMDD region (codons 203–206 of the reverse transcriptase (rt) gene), which is region of the catalytic position of the HBV polymerase(4-6). Three types of mutations are observed in the polymerase gene. There is the M204V associated with L180M (Group I), the M204I alone (Group II) or the M204I with L180M (Group III). TheL180M and M204V mutations act synergistically to increase resistance to lamivudine(7). Resistance to lamivudine is routinely detected by nucleotide sequencing of PCR products(8). Many researchers have found that the state of some HBeAg negative hepatitis B patients is not steady and is even serious(9, 10).Such HBeAg negative patients are in a state of HBV infection in their complete life, and have dynamic HBV replication and continuous liver function injury. This condition is related with mutations in the pre-core (pre-C; nucleotides 1814-1900) and core promoter (CP; nucleotides 1613-1849) regions of HBV genome(11-14). Among these mutations, the pre-C mutation nt1896G/A is common, causing in the early cessation of HBeAg translation(15). The coinciding presence of A1762T and G1764A mutations in the basic core promoter (BCP) region of HBV can lead to a 50%-70% decline in the RNA transcription level in the pre-C region, and thus resulting into reinforcement of viral replication(16-18). Mutation at nucleotide 1896 of the pre-C region (TAG mutation) ends production of the HBeAg, leading to HBeAg-negative CHB, while mutations in BCP region at nucleotides 1762 and 1764 may play a role in HBeAg clearance(10, 19, 20).

The pre-C and BCP mutations also appear to influence response to interferon treatment(21, 22). Testing for these mutations may therefore have prognostic value. Among the many factors that may contribute to pathogenesis and carcinogenesis, scientists have found that A1762T/G1764A double mutation in HBV genome is a predictive biomarker for Hepatocellular carcinoma (HCC) development(18). This mutation is involved in the mechanism of infection with hepatitis B e antigen (HBeAg)-negative virus. A1762T/G1764A double mutation is more prevailing in HBV genotype C, and correlates with increased replication capacity(23). Furthermore, HBV genotype C with1762T/1764A mutations is associated with a higher risk of HCC, and this association is independent of serum HBV viral load(24). Currently, A1762T/G1764A mutation is routinely detected by nucleotide sequencing of PCR products(25). Other mutation detection methods for HBV drug resistance are available or under development, such as restriction fragment length polymorphism, mutation-specific real-time PCR, oligonucleotide microarray, Tetra-ARMS PCR, High resolution melt (HRM) curve analysis and next-generation sequencing (NGS)(8, 26, 27). Each of these methods have their own merits and demerits in terms of time required for analysis, cost incurred for testing, sensitivity and specificity. The present study adopted SNaPshot method mediated by multiplex PCR for detection of each of these significant mutations in single tube reaction.

Our aim here was to develop a rapid, cost-effective and reproducible method for simultaneous detection of major HBV drug resistance mutations (L180M and M204V/I),basal core promoter mutation (A1762T/G1764A) and pre-core mutation (G1896A) by employing multiplex primer combined PCR amplification and subsequent minisequencing (SNaPshot) technique (multiplex primer extension and capillary electrophoresis).

MATERIALS AND METHODS

Study samples:

This study examined a total of 50 blood serum samples from chronic HBV infected patients visiting Molecular Diagnostic Lab of geneOmbio Technologies Private Limited (Pune, India) during March 2012 to December 2016. HBeAg positive (ELISA test) CHB patients having detectable viral load on COBAS Taqman 48 HBV monitor test were included in these 50 samples reference panel. HIV-1 or HCV co-infected patients were excluded. Twenty uninfected control samples were also collected for validation purpose. All procedures conformed to the ethical guidelines of the 1975 Helsinki Declaration. Samples were de-identified and none of

the personal information related to patients was disclosed. This study was approved by Institutional Review Board (IRB) at geneOmbio Technologies, Pune, India. (Approval No. IRB/2012/G-011). The serum samples were collected and stored at -40°C until use.

Multiplex PCR primers and SNaPshot probes:

Primer designing for multiplex PCR and SNaPshot probes was performed using HBV full length sequences of different genotypes (Genbank database) and primer design tool Primer3v0.4.0 available online at http://bioinfo.ut.ee/primer3-0.4.0/primer3/. Primers were designed for amplification of HBV DNA covering drug resistance mutations located at 180th and 204th amino acid positions in polymerase gene. Multiplex PCR primers for amplification of region covering 1762/1764 and 1896 nucleotide positions in BCP and Pre-C gene region respectively were also designed. SNaPshot probes for detection of the selected five mutation locations were designed to anneal on the sense or antisense strand immediately adjacent to the mutation site as detailed in Table 1. Each probe for drug resistance mutation was synthesized with a different length of poly (dT) tail to allow separation of SNaPshot products on the basis of size. SNaPshot probes of BCP and Pre-C mutation were not tagged with poly (dT) tail as the length of primer sequence without tail was sufficient to differentiate probes during capillary electrophoresis.

Standard clones:

Clinical samples with chronic HBV infection having previously identified drug resistance mutation by standard sequencing assay were used for preparation of standard plasmid. HBV DNA was isolated by Roche HiPure Viral Nucleic Acid kit (Roche Diagnostics). PCR was performed in a 2720 thermal cycler and newly designed PCR primers in two separate tubes for pol and BCP – Pre-C genes using following conditions: 94°C for 5 min, followed by 94°C for 30 s, 55°C for 30 seconds, and 72°C for 30 seconds for 35 cycles, with a final extension at 72°C for 5 min. The PCR products were purified (Wizard DNA purification kit, Promega) and cloned into the pGEM-T Easy Vector System (Promega, Madison, WI, USA). The mutations in plasmid clones were confirmed by sequencing using BigDyeTerminator v3.1 cycle sequencing kit on 3130 Genetic analyzer (Applied Biosystems). The sequences that carried the L180M, M204V and M204I mutation from pol gene were selected as standard plasmid clones. In second amplicon, A1762T/G1764A double mutation clone along with another clone having G1896A mutation in Pre-C was selected. Wild type sequence containing clone was also identified and further used for standardization of SNaPshot method.

Multiplex PCR and SNaPshot analysis:

Multiplex PCR was performed in a volume of 25 µl containing 1x PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.5 µM of each primer, 1 unit Taq DNA polymerase (Thermo Fisher Scientific) and five microlitre of template DNA. Thermal cycler conditions were: 95°C for 5 min, 35 cycles of 95°C for 30 sec, 55°C for 30 sec, 72°C for 30 sec and final extension for 10 min at 72°C. Multiplex PCR products were checked for quality and concentration by running 5 µl in 2% agarose-TBE gels. The remaining PCR products were treated with ExoSAP-IT reagent (Affymetrix). For validation purpose, singleplex PCR was performed using both the primer pairs separately to amplify DNA from study samples. These amplicons were sequenced using Sanger's method with the BigDye terminator v3.1 cycle-sequencing kit and ABI 3130 Genetic analyzer. SNaPshot analysis was performed using an Applied Biosystems SNaPshot Multiplex Kit. Nine microliter SNaPshot reaction containing 2.5 µl of SNaPshot Multiplex Ready Reaction Mix, 1x BigDye sequencing buffer, 1 µl of probe mix (consisting of 0.75 µM of each probe) and 2 µl of ExoSAP-IT-treated multiplex PCR product was set for each sample. Extension reactions were performed in a 2720 thermal cycler and consisted of 30 cycles of denaturation at 95°C for 10 sec, annealing at 50 °C for 5 seconds and extension at 60 °C for 30 seconds. Labelled extension products were treated with shrimp alkaline phosphatase (1 unit per sample) then diluted 1 in 10. One microliter of the diluted extension product was mixed with 9.5 µl of HiDi™ formamide and 0.5 µl of Genescan-120LIZ size standard (Applied Biosystems). Products were denatured at 95°C for 3 minutes in a 2720 thermal cycler. The amplicons were separated using an ABI PRISM 3130 Genetic Analyzer with a 36 cm length capillary and POP-7™ polymer. Capillary electrophoresis analysis was performed using GeneMapper 3.7 Software.

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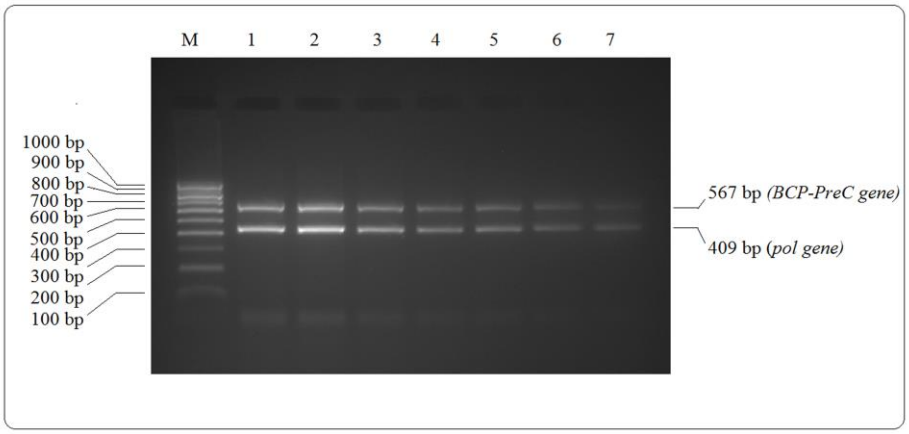
RESULTS

HBV DNA amplification:

Multiplex PCR primers for two different regions in HBV genome simultaneously amplified pol and BCP-Pre-C genes generating two distinct fragments of length 409 and 567 bp respectively **(Figure 1)**. Each of the standard clone amplified successfully. Thirty eight out of fifty DNA samples from validation panel were effectively amplified using the multiplex PCR primers. Viral load of the non-amplified samples was less than 500 copies/mL as determined by COBAS TaqMan 48 HBV test (Roche Molecular Diagnostics).

Figure 1: Multiplex polymerase chain reaction of two genes simultaneously in a single tube reaction resulting in 409 bp and 567 bp amplicons. Lane M: 100-1000bp DNA ladder, Lane 1-7: Study samples.



Strategies for detection of each mutation:

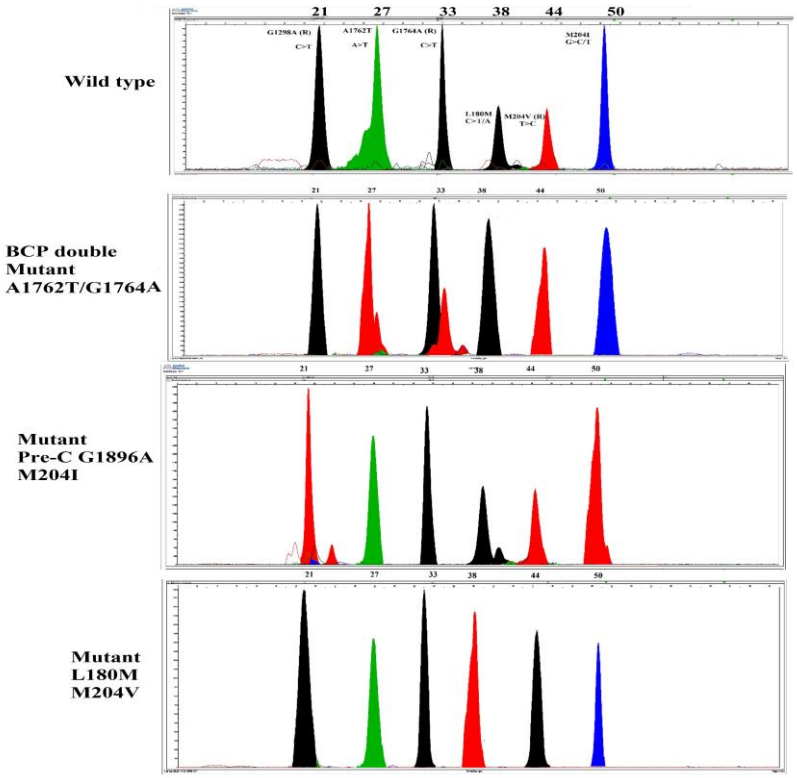


Figure 2: SNaPshot electropherograms obtained from four different samples that show all representative wild type and mutant loci. The X-axis represents the size of the mini-sequencing products (nucleotides); the Y-axis represents relative fluorescence units (RFUs). STD: GS120 LIZ size standard.

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Probe BCP_A1762T recognized the HBV locus 1762, while probes BCP_G1764A and Pre_C_G1896A, recognized the HBV loci 1764 and 1896, respectively. The single base extension of these three probes produced 27, 33 and 21 bp fragments, respectively **(Table 1, Figure2)**. For drug resistance mutations, Probe Pol_L180M recognized mutation at 180th amino acid (CTG→ATG) in pol gene while probes Pol_M204V and Pol_M204I detected HBV pol amino acid 204 changing to Valine (ATG→GTG) and Isoleucine (ATG→ATC, ATT), respectively. The single base extension of these three probes produced 38, 44 and 50 bp fragments, respectively **(Table 1, Figure 2)**.

Table 1: Mulitplex PCR primers for amplification of two different fragments from HBV genome and SNaPshot probes for detection of mutations in the amplified fragments

Gene	Multiplex Primers (5'-3')	DIR[a]	LEN[b]	Amplicon Size (bp) (Locus)	Tm (°C)	%GC
Polymerase	CTGTATTCCCATCCCATCATC	F	21	409 (598-1006)	59	48
	GACCCACAATTCGTTGACATAC	R	22		58	45
BCP-Pre-C	GGGACGTMCTTYGTYTACGTC	F	21	567 (1411-1977)	53	48
	GAAGGAAAGAAGTCAGAAGGC	R	21		52	48
Mutation Locus/ Probe Name	**SNaPshot Primers(5'-3')**	**DIR[a]**	**LEN[b]**	**SNP[c]**	**Tm (°C)**	**%GC**
BCP_A1762T	AGGAGYYGGGGGAGGAGATTWGRTTAA	F	27	A/T	60	48
BCP_G1764A	CCAATTTATGCCTACAGCCTCCTARTACAAAGA	R	33	C/T[d]	62	42
Pre_C_G1896A	TACGGGTCAATGTCCATGCCC	R	21	C/T[d]	57	56
Pol_L180M	TTTTTTTTTTTTTTTTTTTTGCCTCAGTCCGTTTCTC	F	38	C/T/A	58	59
Pol_M204V	TTTTTTTTTTTTTTTTTTTTTTTTCCCAATACCACATCATCCA	R	44	T/C[d]	58	47
Pol_M204I	TTTTTTTTTTTTTTTTTTTTTTCCCCCACTGTTTGGCTTTCAGTTATAT	F	50	G/C/T	61	40

[a]Direction of primers
[b]Length of primer
[c]Nucleotide changes that can be detected by SNaPshot probes
[d] As the probes is in reverse orientation, the reverse complement base is detected.

SNaPshot analysis optimization:

The probe length (21, 27, 33, 38, 44 and 50 bp), its concentration (0.75 µM) and the template concentration were optimized to ensure consistency in the height and position of the peaks representing the five different types of extension products. DNA template concentration ranging from 5ng-20ng/µl showed a significant linear regression with the peak height (r=0.995).

Specificity and sensitivity:

This novel multiplex SNaPshot method was validated by analysis of the HBV mutant standard plasmid clones identified using Sanger sequencing method. For each drug resistance mutation locus as well as mutations in BCP and Pre-C region SNaPshot method showed hundred percent concordance with Sanger sequencing method. Multiplex PCR of negative control samples showed no amplification, thus ruled out the chances of false positive cases, proving the method as highly specific for HBV DNA amplification. The sensitivity of the SNaPshot assay was assessed by analysis of the diluted HBV DNA samples, wherein it was observed that the multiplex SNaPshot method is applicable to samples having more than 500 HBV DNA copies/ml. Diluted HBV DNA with less than 500 copies showed no amplification in multiplex PCR, hence could not be subjected to further SNaPshot analysis. This was in concordance with the earlier results in which 38 out of 50 samples were amplified having viral load greater than 500 copies/mL.

Comparison of the SNaPshot assay and DNA sequencing:

The SNaPshot assay was evaluated using clinical serum samples from patients identified with chronic HBV infection. All samples were analyzed by the SNaPshot assay and DNA sequencing. A comparison of the results obtained using the SNaPshot assay and DNA sequencing demonstrated 100% (38/38) sensitivity and validity of the SNaPshot assay**(Table 2)**. The accuracy of the SNaPshot assay for detection of mixture of mutants was found to be equivalent to that of direct DNA sequencing [100% (3/3) vs 100% (3/3)]

Table 2: Comparison of the results of SNaPshot method and sequencing for detection of HBV mutations

Parameters	SNaPshot	Sequencing
Number of clinical specimen	50	50
Type of amplification	Multiplex PCR	Singleplex PCR
Number of samples amplified	38 (76%)	38 (76%)
Mean HBV viral load, log_{10} IU/mL by CTM48[e]	4.23 $\pm$ 1.83	4.23 $\pm$ 1.83
BCP A1762T	6 (15.8%)	6 (15.8%)
BCP G1764A	3 (7.9%)	3 (7.9%)
Pre-C G1896A	4 (10.5%)	4 (10.5%)
Pol L180M	4(10.5%)	4(10.5%)
Pol M204V	1 (2.6%)	1 (2.6%)
Pol M204I	2 (5.3%)	2 (5.3%)
Wild type[f]	25 (65.8%)	25 (65.8%)

[e]Viral load by Cobas Taqman 48 HBV kit converted to log_{10} IU/mL

[f]Two samples harbored A1762T/G1763A double mutations and M204V/I mutants were obtained in same samples that has L180M mutation hence completely wild type sample number for these mutant loci is higher than the sum of mutants.

DISCUSSION

In the present study, a SNaPshot assay was established for the simultaneous detection of important HBV mutation in clinical specimens. The methodology employed for this assay is also regarded as a mini-sequencing technology(28). The reaction mixture consisted of a polymerase, four fluorescent-labeled dNTPs and extension probes. The position of the nucleotide base at the termination site is associated with the mutation, and this is readily detectable by differential fluorescence peak analysis. The method detects six different type of mutations located at five different positions in HBV genome in a single run experiment. This has advantages over the technique used by Wang et al(29) as they were only able to detect BCP and pre-C regions mutations, whereas in SNaPshot method we have multiplexed it to include drug resistance mutation in pol gene.

The results of the present study demonstrate that the SNaPshot assay offers a rapid, robust and highly reliable alternative to traditional DNA sequencing methods for simultaneous detection of drug

resistance, basal core protein and Pre core region mutations. In another study, scientists have used SNaPshot method for detection of few mutations in HBV YMDD codon region that included 13 patients. The 13 samples were simultaneously tested with SNaPshot and DNA sequencing, the same results were obtained. The study reported thatSNaPshot method showed high specificity(30).Due to use of multiplex technology this method effectively reduces the cost of PCR amplification as reported in few earlier studies that used multiplex PCR for various applications (31-33). Further, this method enables detection of all six types of mutations in single run which in turn reduces cost equivalent to two more Sanger sequencing reactions.

In summary, we have developed a highly sensitive Simple- Probe-based multiplex PCR assay for the detection of essential HBV mutations. The unique assay design reduces the analysis time and cost and helps in identifying resistant antivirals caused due to viral genome polymorphism. This assay platform may be useful in mutation detection for all patients on treatment, such that treatment can be effectively directed to drugs for which the virus is susceptible. This approach for highly sensitive mutation detection may also be applied to identification of other clinically significant mutations in HBV covering other mutant loci.

CONCLUSION

In conclusion, the present study showed that the SNaPshot method is able to be used for the simultaneous analysis of the mutations at different sites in HBV genome having huge clinical significance in patient treatment decision. The sensitivity and accuracy of this technique was shown to be comparable to that of the sequencing method. The SNaPshot method is limited by its potential for the detection of known mutations only. However, it gives significant information about presence or absence of mutations along with the mixture or mutant strains present in a particular sample. Thus, the present study provides the basis of a novel technique that is suitable for further development in HBV patient's molecular analysis.

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CONFLICT OF INTEREST: None to declare.

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