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Hepatitis B virus (HBV) genome detection and genotyping in virally suppressed patients using nested polymerase chain reaction-based Sanger sequencing

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ABSTRACT

Hepatitis B virus (HBV) genotypes have important clinical implications. Current genotyping methods are less sensitive in patients with ultra-low HBV viral load. We report a highly sensitive and specific nested polymerase chain reaction (PCR) assay for genotyping patient HBV. Total DNA derived from plasma of 14 (HBsAg+ and/or HBsAg-) HBcAb+ patients was used for HBV-specific nested PCRs targeting the preC/C, X/BCP/preC, and surface regions. All patients were treated with long-term nucleos(t)ide analogues (NAs), and 12/14 have undetectable viremia (clinical PCR: sensitivity >10 IU/mL). Surface amplicons were sequenced, aligned with reference genomes, and used in phylogenetic tree construction to determine genotype. HBV DNA was detected in 14/14, including 3 occult (HBsAg-/HBcAb+) cases. Genotypes identified were 6/14 B, 6/14 C, and 2/14 D. This assay in virologically suppressed patients may be useful for future studies requiring genotype prior to assessment of immunomodulatory and/or direct acting anti-viral therapeutics in patients on potent NAs.

1. Introduction

There are an estimated 240 million hepatitis B virus (HBV) surface antigen (HBsAg) positive chronic hepatitis B (CHB) carriers worldwide who are at risk of severe liver complications including cirrhosis, liver failure, or primary liver cancer (i.e. hepatocellular carcinoma, HCC) (Schweitzer et al., 2015). Current first-line anti-viral therapies (i.e. nucleos(t)ide analogues) target the error-prone reverse transcriptase polymerase of HBV. Although these therapies lack the ability to directly target the covalently closed circular DNA (cccDNA) of HBV, they are effective in significantly reducing viral load or DNA to low levels.

Occult HBV infection is characterized by HBsAg negativity, but with detectable HBV DNA by using highly sensitive assays in serum, peripheral blood mononuclear cells or in liver (Raimondo et al., 2008). Both HBsAg positive individuals with low-level (ie, HBV DNA <2000 IU/mL) as well as those with occult HBV remain at continual risk of liver disease. Numerous studies have suggested that risk of primary liver cancer (HCC) in low-level HBV CHB carriers is not negligible and remains higher than those without HBV infection, particularly when additional liver co-morbidities, such as cirrhosis, are present (Chen et al., 2010; Fung et al., 2007; Kim et al., 2017; Sinn et al., 2015). Occult HBV is correlated with liver damage, cirrhosis, HCC severity, and characterized viral oncogenic risk factors (preS mutations) suggesting contributing roles towards liver cancer development (Coppola et al., 2016; Pollicino et al., 2004; Squadrito et al., 2006). These clinical implications are further supported by reports that individuals with occult HBV are also subject to viral integration, which has inherent oncogenic risk factors and can affect oncogenes and tumor suppressor genes (Murakami et al., 2004; Saitta et al., 2015). In addition, we have previously reported that single nucleotide polymorphisms (SNPs) in the HBV basal core promotor/X gene region, which are associated with liver cancer and end-stage liver disease, persist in low-level and occult HBV patients even many years post-liver transplantation (Lau et al., 2018).

Due to error-prone replication, the HBV exists as a quasi-species population. There are 10 HBV genotypes, labeled A to J, that have been identified based upon a divergence of >7.5% in the full HBV genome sequence (Tong and Revill, 2016). The distribution of HBV genotypes is geographically distinct with certain genotypes endemic in specific regions. For example, genotypes B and C are predominant in East Asia whereas genotype D is prevalent in central Asia and the Mediterranean. However, regions with larger influxes of immigrants, such as Canada,

Abbreviations: CHB, Chronic hepatitis B; HBsAg, Hepatitis B surface antigen; HBV, Hepatitis B virus; HCC, Hepatocellular carcinoma; IQR, Interquartile range; REB, Research ethics board; SNP, Single nucleotide polymorphism; VGE, Virus genome equivalents.

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are prone to a large diversity of HBV genotypes reflective of the origin of the migrants (Congly et al., 2013; Osiowy et al., 2015). HBV genotypes may be associated with distinct clinical outcomes and response to therapy. For example, patients chronically infected with genotype A have improved responses to IFN-based anti-HBV therapies, those with genotype B are linked to earlier seroconversion to HBeAg negativity, and genotype C is highly associated with hepatocellular carcinoma development (Lin and Kao, 2015; Rajoriya et al., 2017). These clinical differences emphasize the value of accurate genotype determination allowing a tailored approach to management of hepatitis B (i.e. precision medicine), particularly in genotypically and ethnically diverse regions such as Canada.

A variety of different techniques are available for genotyping of HBV. The gold standard for genotype determination involves sequence and phylogenetic analysis of the full length HBV genome (Guirgis et al., 2010). This approach lacks the large-scale cost-effectiveness and efficiency for regular use in clinical disease management. Further, full genome amplification of HBV is challenging particularly in low viremia CHB carriers. The INNO-LiPa HBV Genotyping kit (Fujirebio US, Inc., Malvern, USA) is commonly used for genotype determination of HBV, and may also be used for determination of drug resistant mutations. This commercial kit utilizes biotin labeled amplicons followed by hybridization with genotype specific probes. However, the conventional INNO-LiPa kit lacks the sensitivity for consistently determining the genotype of low-level or clinically undetectable HBV (i.e. <10 IU/mL) (Qutub et al., 2006). In addition, the cost of the INNO-LiPa kit may restrict usage on a large-scale clinical setting, particularly in developing endemic countries.

Generally, the detection and genotype determination of low-level and occult HBV remains a challenge, particularly in CHB carriers that are successfully treated with potent anti-viral therapies. Thus, in our current study we report the robust use of a nested PCR based technique for accurate detection of HBV DNA in clinically undetectable and/or occult CHB carriers. We adapted the direct and nested PCR of the INNO-LiPa in combination with sequencing and phylogenetic tree analysis for genotyping. We tested a representative cohort of patients with undetectable HBV DNA according to clinical PCR assay, in which HBV DNA was detected in the plasma by in-house nested PCR, and different HBV genotypes were determined.

2. Methods

2.1. Summary of chronic HBV carriers and samples collected

This study was approved by the University of Calgary conjoint health research ethics board, CHREB (Ethics ID 16636). All subjects were recruited from the Calgary Liver Clinic, University of Calgary and provided informed written consent to participate according to the guidelines of the 1975 Declaration of Helsinki. Inclusion criteria selected for CHB carriers receiving potent anti-HBV therapy with undetectable HBV DNA in plasma according to clinical PCR assay. Approximately 50 ml of whole blood was collected in EDTA vacutainers from patients for isolation of plasma in addition to relevant clinical and treatment data obtained at the time of sample collection. Viral load measurements closest to date of sample collection, (minimum of 3 months before or after) were collected. HBV DNA viral load was quantified according to clinical PCR assay: Abbott m2000 RealTime HBV Viral Load (sensitivity 10 IU/mL or ~50 virus genome copies/mL).

2.2. Isolation and detection of HBV DNA from plasma

Total DNA was isolated from 500 μL of plasma using standard phenol chloroform extraction with ethanol precipitation and resuspended into 50 μL of water. 10 μL of extracted DNA was used for detection of HBV by nested PCR using primers specific for the HBV preC/C, X/BCP/preC, and surface (S) genomic regions (Table S1). 5 μL and 2 μL of the direct round PCR products were used as template for the nested round to account for potential inhibitory effects of excessive template in the second reaction. The X/BCP/preC and surface genomic region PCR protocols were adapted from Takahashi et al. and the INNO-LiPA HBV genotyping assay, respectively (Takahashi et al., 1995). The preC/C and X/BCP/preC nested PCR techniques have been previously reported (Lau et al., 2018; Coffin et al., 2011, 2015). The DNA templates were added to a PCR master mix consisting of 5 μL of 10X PCR Buffer (Takara Bio USA Inc., Mountain View, USA), 4 μL of 10 mM dNTPs (Takara Bio USA Inc.), 1 μL of each primer at 10 μM, and 0.25 μL of TaKaRa Taq DNA polymerase HotStart (Takara Bio USA Inc.) in a final 50 μL reaction volume followed by amplification with a standard thermocycler (T100 Thermal cycler, Bio-Rad Laboratories [Canada] Ltd., Mississauga, CAN) (Table S2). PCR products were analyzed with 1% agarose gel electrophoresis. Assay sensitivities for HBV DNA detection with preC/C and X/BCP/preC nested PCRs were <10 virus genome equivalents (vge)/mL of plasma (Lau et al., 2018; Coffin et al., 2011). Surface region nested PCR sensitivity was assessed as per methods described below.

DNA extraction and PCR set-up were performed in separate locations to minimize carryover of HBV sequences. Negative controls included "mock" DNA isolations with water and plasma from healthy HBV negative individuals under strict conditions to exclude possible environmental risk of contamination. Ultra-low PCR positive control included plasmid DNA containing one full genome copy of the HBV genome constructed in-house.

2.3. Evaluation of the sensitivity of the HBV surface genomic region nested PCR

Plasmid DNA containing a single full genome copy of the HBV genome was constructed in-house. Briefly, HBV full genome was amplified from phenol-chloroform DNA isolated from a high viral load clinical sample (>10^8 IU/mL) with high-fidelity Phusion DNA polymerase (New England Biolabs, Whitby, Ontario, Canada) as per manufacturer's protocols. HBV FG P1 and P2 primers for amplification (Tables S1, S2) were derived from Günther et al. (1995). PCR amplicons were purified from a 1% agarose gel followed by digestion with HindIII (New England Biolabs) and ligated with T4 DNA ligase into the cloning vector pUC19 which was similarly prepared. Ligations were transformed into TOP10 *E. coli* chemically competent cells (Invitrogen, Carlsbad, USA) and successful clones were identified and confirmed via PCR and sequencing respectively with standard M13 primers (Table S1).

Following plasmid isolation with the GenElute plasmid miniprep kit (Millipore Sigma, Oakville, Ontario, Canada), cloned HBV copy number was determined via an established in-house quantitative PCR targeting the HBV surface genomic region (Gao et al., 2017). 2 sets of 10-fold serial dilutions of the plasmid from 10^3 to 10^{-2} and 2×10^3 to 2×10^{-2} copies/ μL were prepared. 1 μL of each dilution was used as template in HBV surface genomic region nested PCR as described above in 3 separate experimental replicates. In addition, to simulate our described extraction and PCR technique, 2 sets of 10-fold serial dilutions of the plasmid from 10^3 to 10^{-2} and 5×10^3 to 5×10^{-2} IU/ μL were prepared. Copies/ μL calculated in-house were converted to IU/ μL using a conversion factor of 5 copies = IU. 1 μL of each dilution was used to inoculate 500 μL of healthy (ie., HBV negative) plasma followed by total DNA extraction and HBV surface genomic region nested PCR as described above in 3 separate experimental replicates. Healthy plasma used in plasmid inoculation was also subjected to DNA extraction and nested PCR as a negative control.

PCR mastermix preparations were performed in a dedicated location to minimize carryover of HBV sequences and environmental contamination. Negative control of water was used to exclude possible environmental risk of contamination. PCR positive control consisted of 10^7 copies of the house-made plasmid DNA.

2.4. Determination of HBV genotype and phylogenetic tree analysis

HBV surface genomic region PCR amplicons from clinical samples were agarose gel purified (QIAquick gel extraction kit, Qiagen, Hilden, DEU) followed by bidirectional in-house Sanger sequencing (University of Calgary Core DNA services, Calgary, Alberta, Canada) with the nested PCR primers (HBPr75 and HBPr94). Sequencing results were aligned with reference HBV genomes representative of the major genotypes (Table S3) using MAFFT version 7 (https://mafft.cbrc.jp/alignment/server/). Phylogenetic analysis was performed using the MEGA software version 7 (Kumar et al., 2016) to construct a maximum likelihood tree with the Kimura-2-parameter substitution method and 1000 bootstrapping replicates. Ambiguous results from phylogenetic analysis were confirmed with the NCBI genotype sequencing tool for HBV (https://www.ncbi.nlm.nih.gov/projects/genotyping/formpage.cgi). Sequence alignments were also used for identification of SNPs implicated in HBV immune escape and drug resistance.

3. Results

3.1. Summary of clinical and virological data (Table 1)

In total, 14 CHB carriers (4 females; median age 55.5 [IQR: 44–65.75], 1 African, 13 Asians) were enrolled in this study (Table 1). All subjects recruited received potent first-line nucleos(t)ide analogues for anti-HBV therapy (3 entecavir, 11 tenofovir disporoil fumurate). Two cases (21–2 and 33–2) with detectable levels of HBV (>10 IU/mL) and clinical genotyping results via the INNO-LiPa kit were included for validation of our genotyping methodology. Our cohort includes 12 representative cases with clinically undetectable HBV on nucleos(t)ide analog therapy classified into 3 groups according to liver disease sequelae: (1) 4/12 (33.3%) were liver transplant recipients for HBV-related liver failure. 1/4 transplant recipients subsequently had viral breakthrough (HBsAg positive recurrence) with the remaining 3/4 continually identified as occult HBV infection with negative HBsAg). (2) 5/12 (41.7%) CHB carriers were cirrhotic of which 3 have progressed to HCC. (3) The remaining 3 of 12 (25%) clinically undetectable cases were treated based on guidelines for active hepatitis B but have no known HBV-related liver co-morbidities.

3.2. Low level HBV DNA is detectable by the surface genomic nested PCR

To evaluate the sensitivity of our surface genomic nested PCR, serial dilutions of a house-made plasmid containing a single copy of the HBV full genome (pUC19-HBV) was amplified. 10^3 to 10^{-2} copies and 2×10^3 to 2×10^{-2} copies of the plasmid HBV (equivalent to approximately 2×10^{-1} to 2×10^2 IU and 4×10^{-1} to 4×10^3 IU, respectively) in log10 serial dilutions were tested. Although, the direct round PCR was insufficient for detection of the HBV DNA in both dilution series, all samples ≥1 copy/reaction were detectable in the second (nested) round of PCR (Fig. S1) thereby demonstrating the high sensitivity of our assay. We further demonstrate the effectiveness of the nested PCR by inoculating healthy plasma with HBV plasmids in serial dilutions (1×10^{-2} to 1×10^3 IU and 5×10^{-2} to 5×10^3 IU). HBV DNA in plasma was reliably detected in 500 μL of plasma with addition of 5 IU of plasmid HBV (approximately 25 copies), although faint bands were consistently detected at inoculation of 1 IU (approximately 5 copies) of HBV genomes (Fig. 1). Similarly, our PCR assay was evaluated in plasma collected from 11 additional CHB carriers with detectable HBV DNA via clinical assays (Abbott m2000 RealTime HBV viral load), but unquantifiable viral load (i.e. <10 IU/mL). 10 of the 11 (90.9%) of these samples were detectable by the HBV surface genomic region nested PCR (Table 2).

3.3. HBV DNA is consistently detectable in the plasma of clinically undetectable and low-level CHB carriers

We evaluated the surface genomic nested PCR alongside previously established methods of detection used in our laboratory including the preC/C and X/BCP/preC nested PCR (Lau et al., 2018; Coffin et al., 2011, 2015; Gao et al., 2017; Virine et al., 2015). HBV DNA was detected in the plasma of all 14 cases with primers specific for the preC/C, X/BCP/preC, and/or surface genomic regions (Table 3), despite undetectable viral load by clinical PCR assay in 12/14 (85.7%). Nested PCR consensus was observed in 14/14 (100%) cases with amplification by two or more HBV-specific primer sets and in 11/14 (78.5%) cases with amplification by all 3 primer sets which includes 3 HBsAg negative, putative occult hepatitis B cases (case ID# 75, 87, 200). Detection rate was similar in

Table 1
Clinical and virological features of 14 CHB carriers with potent nucleos/tide analog therapy included in our cohort. 12 cases are representative of CHB carriers at various stages of disease with clinically undetectable viremia.

Case ID#	Age/sex	Ethnicity	Viral load (IU/ML)*	Anti-viral therapy	Additional notes
21–2	50 / M	AS	340	TDF	GENOTYPE B+
33–2	57 / M	AS	17,000	TDF	GENOTYPE B+
14	48 / M	AS	U	TDF	HCC CIRRHOSIS
64	65 / M	AS	U	TDF HBIG	POST-LT
75	54 / M	AS	U	TDF HBIG	POST-LT OCCULT HBV
87	32 / F	AF	U	TDF HBIG	POST-LT OCCULT HBV
94–3	44 / M	AS	U	TDF	
113–2	62 / M	AS	U	TDF	CIRRHOSIS
114–2	40 / M	AS	U	ETV	
191	68 / M	AS	U	TDF	HCC CIRRHOSIS
200	28 / F	AS	U	TDF HBIG	POST-LT OCCULT HBV
247	69 / M	AS	U	ETV	
266	73 / F	AS	U	ETV	HCC CIRRHOSIS
311	58 / F	AS	U	TDF	CIRRHOSIS
TOTAL	Median age = 55.5 4 / 14 F	1 African (AF) 13 Asian (AS)	12 Undetectable (U)	3 Entecavir (ETV) 11 Tenofovir (TDF) 4 Hepatitis B immune globulin (HBIG)	

* Determined by clinical PCR assay, Abbott m2000 RealTime HBV Viral Load (sensitivity >10 IU/mL; 1 IU/mL = ~5.26 copies/mL)
\+ Genotype determined in clinic using INNO-LiPa assay.

the X/BCP/preC and surface genomic nested PCRs with HBV DNA amplified in all cases.

3.4. Determination of HBV genotypes and simultaneous identification of clinically important SNPs present in the plasma and PBMC

HBV genotype of the amplified surface genomic region was determined by analysis of phylogenetic clusters, thereby revealing 6 genotype B, 6 genotype C, 2 genotype D (Table 2, Fig. 2). The primary genotypes detected, B and C, correlates directly with our cohort demographics which is pre-dominantly (92.9%) individuals of Asian ethnicity. Genotypes determined of the two clinically detectable cases (ID# 21–2 and 33–2) directly matched the clinical results. As our sequencing region overlaps with the HBV surface and polymerase genes, we simultaneously evaluated the presence of SNPs with clinical importance including the immune escape (sG145R) and nucleos/tide analogue resistant variants (rtI169T, V173 L, L180 M, A181V/T, T184S/A/I/L/G/C/M, A194T, T184G/S, S202I/G, M204I/V). No SNPs were identified in the analyzed samples.

4. Discussion

The HBV genotype may be clinically important in determining disease management and prognosis (Rajoriya et al., 2017). Common genotyping methodologies of full genome sequencing and the INNO-LiPa kit are subject to certain limitations, particularly with detection and genotype determination in patients with undetectable (<10 IU/mL) HBV viral load (Guirgis et al., 2010; Qutub et al., 2006). In the

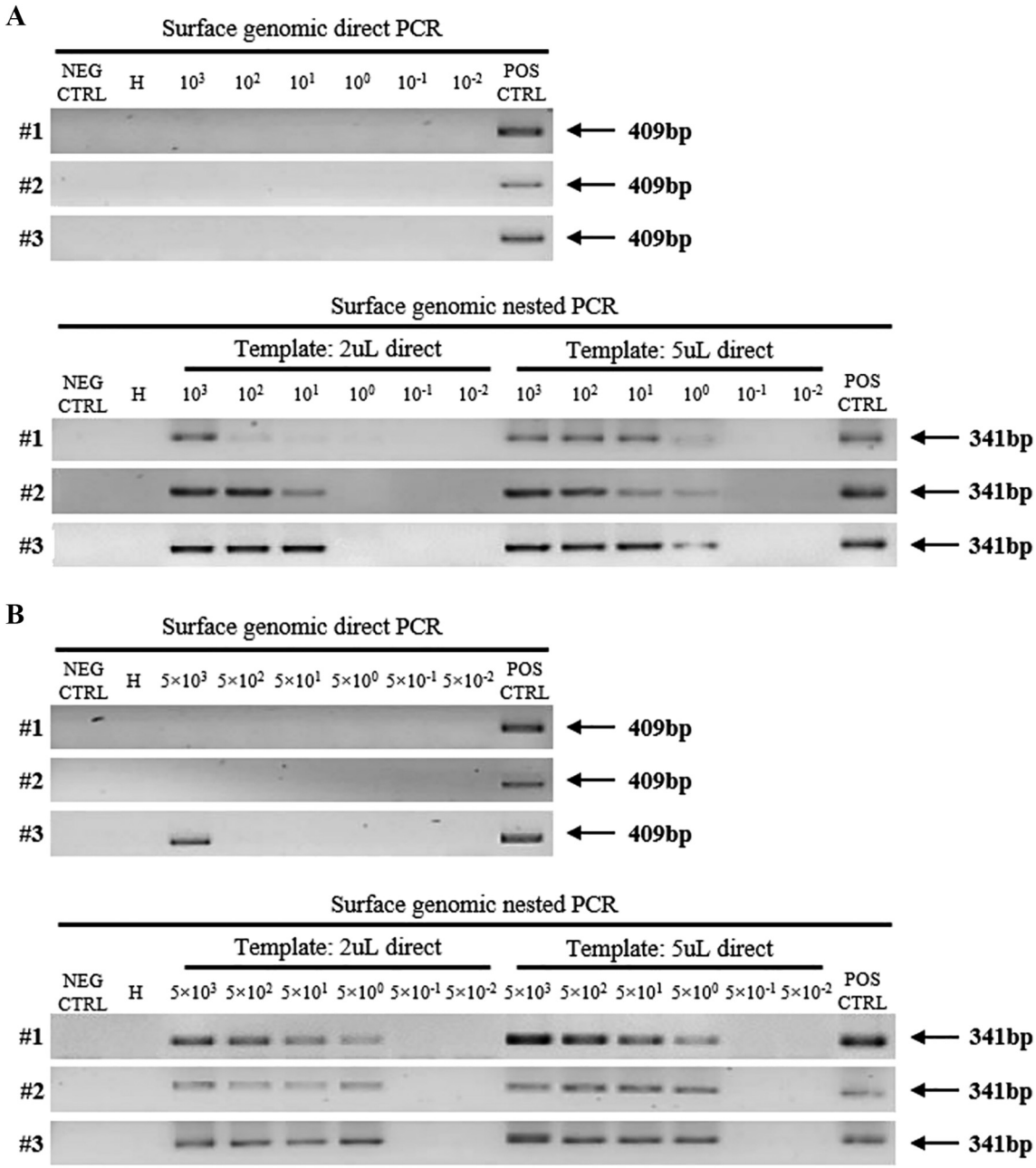

Fig. 1. Surface genomic region nested PCR was evaluated by inoculating healthy HBV negative patient plasma with 1× HBV genome containing plasmid in 3 different sets of experimental replicates. Total DNA extracted via phenol-chloroform was subjected to direct and nested PCR for HBV surface genomic region. PCR products from the **A.** plasmid serial dilution 1 (10^3 to 10^{-2} IU/extraction) and the **B.** plasmid serial dilution 2 (5×10^3 to 5×10^{-2} IU/extraction) were analyzed on a 1% agarose gel. 2 μL and 5 μL of the direct round PCR was used as template in the nested round PCR. Negative included a no template control (ie., water) and healthy (H) HBV negative plasma. 10^7 copies of the HBV full genome plasmid were utilized as a PCR positive.

Table 2
Plasma from 11 CHB carriers with detectable HBV viremia, but unquantifiable viral load as determined by clinical PCR assay (Abbott m2000 RealTime HBV viral load: sensitivity >10 IU/mL) was evaluated by nested PCR targeting HBV preC/C, X/BCP/preC, and surface genomic regions.

CASE ID#	PREC/C Nested PCR	X/BCP/PREC nested PCR	Surface genomic nested PCR
2–2	+	+	+
6–2	+	+	+
13–2		+	
24–2		+	+
83		+	+
261	+	+	+
265	+	+	+
302	+	+	+
313	+	+	+
349	+	+	+
352	+	+	+
TOTAL	8	11	10

current study, we evaluated plasma samples from 14 patients, 3 of whom are classified as occult HBV with HBsAg negative serology. All cases included in this study received potent nucleos(t)ide analogue anti-HBV therapy and 12/14 showed undetectable viral load according to clinical PCR assay. Despite the clinical viral suppression, we amplified HBV DNA in all patient samples. All 14 cases were consistently detected with at least two of the three HBV-specific nested PCRs. We have previously utilized the preC/C and X/BCP/preC nested PCRs for detection of persistent and low level HBV in diverse settings including HIV/HBV co-infected, CHB carriers with anti-viral therapies, and post-liver transplant individuals (Lau et al., 2018; Gao et al., 2017; Lee et al., 2015).

The clinical implications of chronic low-level HBV replication have yet to be fully elucidated. Studies have suggested that this phenomenon is correlated with a variety of hepatic and extrahepatic complications including cirrhosis, hepatocellular carcinoma, lymphoma, and leukemia (Chen et al., 2010; Fung et al., 2007; Kim et al., 2017; Sinn et al., 2015; Tajima et al., 2016; Zhang et al., 2017). Current clinical methods of HBV viral load quantification and detection are limited by a lower detection limit of 6 IU/mL (~30 vge/mL) via the COBAS TaqMan assay. We demonstrate a HBV surface genomic nested PCR technique capable of detecting ultra-low levels of HBV DNA within a sample. Our study was limited by the inability to accurately quantify HBV DNA at very low levels of plasmid (Fig. 1), thereby hindering identification of a lower limit of detection for our assay. Furthermore, to convert between copies of plasmid calculated in-house and IU/mL, we used the approximate factor of 5 thereby introducing potential error into our analysis of the surface genomic region nested PCR (Saldanha et al., 2001). Nonetheless, in combination with direct Sanger sequencing, the PCR strategy utilized in this study was effectively and efficiently used for all clinical samples tested. However, a major caveat of this highly sensitive method of HBV detection is the concern of contamination. In our current study, we used strict precautions to exclude environmental contamination and HBV DNA carryover including dedicated working areas, equipment, and reagents. Subsequent application of this technique in a clinical or reference laboratory setting should take these concerns into careful consideration to ensure accurate and reliable results.

Table 3
Detection of HBV DNA in the plasma of 14 CHB carriers of which 12 have clinically undetectable viremia with nested PCR targeting HBV preC/C, X/BCP/preC, and surface genomic regions. Surface genomic region amplicons were bidirectionally sequenced for phylogenetic analysis to determine genotype.

CASE ID#	Viral load (IU/ML)*	PREC/C nested PCR	X/BCP/PREC nested PCR	Surface genomic nested PCR	Genotype
21–2	340	+	+	+	B
33–2	217	+	+	+	B
14	U	+	+	+	C
64	U		+	+	C
75	U	+	+	+	C
87	U	+	+	+	D
94–3	U	+	+	+	B
113–2	U	+	+	+	D
114–2	U	+	+	+	B
191	U	+	+	+	C
200	U	+	+	+	B
247	U		+	+	C
266	U	+	+	+	C
311	U		+	+	B
TOTAL	12 U	11	14	14	6 B / 6 C / 2 D

* Determined by clinical PCR assay, Abbott m2000 RealTime HBV Viral Load (sensitivity >10 IU/mL; 1 IU/mL = ~5.26 copies/mL).

Our study cohort was mostly Asian infected by either vertical or early childhood (horizontal) infection, but we were able to successfully determine genotype determination in patients with diverse ethnic backgrounds with HBV genotypes (from B to D). This diversity of HBV genotypes is expected and in line with previous reports of genotype distribution in immigrant-rich nations, including Canada (Congly et al., 2013; Osiowy et al., 2015). Additionally, we demonstrate the effectiveness of this genotyping technique in a wide variety of CHB disease stages including non-cirrhotic, cirrhotic, liver cancer, and post-liver transplant in clinically undetectable cases. We have also previously applied this genotyping methodology to characterize HBV persistence in a larger post-liver transplant cohort (Lau et al., 2018). In 2 cases (ID# 21–2 and 33–2), the HBV genotype was known pre-treatment (determined by clinical INNO-LiPa), and our reported in-house genotyping analysis produced identical results.

Appropriate detection and genotype analysis of HBV is of increasing importance for precision medicine, particularly with management of CHB and novel developments of a variety of anti-HBV immunomodulatory and direct acting therapeutics. Differences in HBV genotype may impact the progression of CHB or influence the development of liver co-morbidities. For example, the increased risk of HCC associated with HBV genotype C is well documented in the REVEAL-HBV study (Chen et al., 2007; Rajoriya et al., 2017; Yang et al., 2008). However, aside from HCC risks, additional effects of viral genotype in disease are poorly understood. With further indications of the role of HBV genotype towards disease progression, patients could be stratified for improved CHB management such as better surveillance of co-morbidities in those with higher risks and specific selection of anti-viral therapies. Previously, it has been demonstrated that HBV genotype correlates with treatment responses to pegylated-interferon (Flink et al., 2006; Janssen et al., 2005). Similarly, clinical success of novel immunomodulatory anti-viral drugs such as the toll-like receptor (TLR) agonists could be affected by HBV genotype. Indeed, Visvanathan and colleagues (Visvanathan et al., 2016) have demonstrated that TLR signaling pathways and expression varies with HBV genotype, thus likely influencing responses to TLR agonist therapies.

The simultaneous identification of common drug resistant mutations is possible albeit none were detected in our cohort. Our strategy for HBV genotype could be adapted in clinical or reference laboratory use, with a standardized protocol and methodology, but requires strict precautions and training of laboratory personnel to avoid contamination. The costs associated with this technique are restrained by the lack of plasmid cloning or full HBV genome amplification as required by current gold standard genotyping. Standard PCR reagents and equipment are sufficient for reliable amplification of the target HBV regions thus limiting requirement for specialized equipment or kits. Free-to-use software similar to those applied in this study are readily available for data analysis of sequence alignments and phylogenetic analysis. The target region amplified coincides with important sites of HBV mutation such as immune escape and drug resistant SNPs which can be simultaneously evaluated thus eliminating multiple work-flows for detection, genotyping, and mutational analysis. In conclusion, we report a sensitive and effective strategy for detecting and genotyping HBV virologically suppressed patients with hepatitis B virus infection.

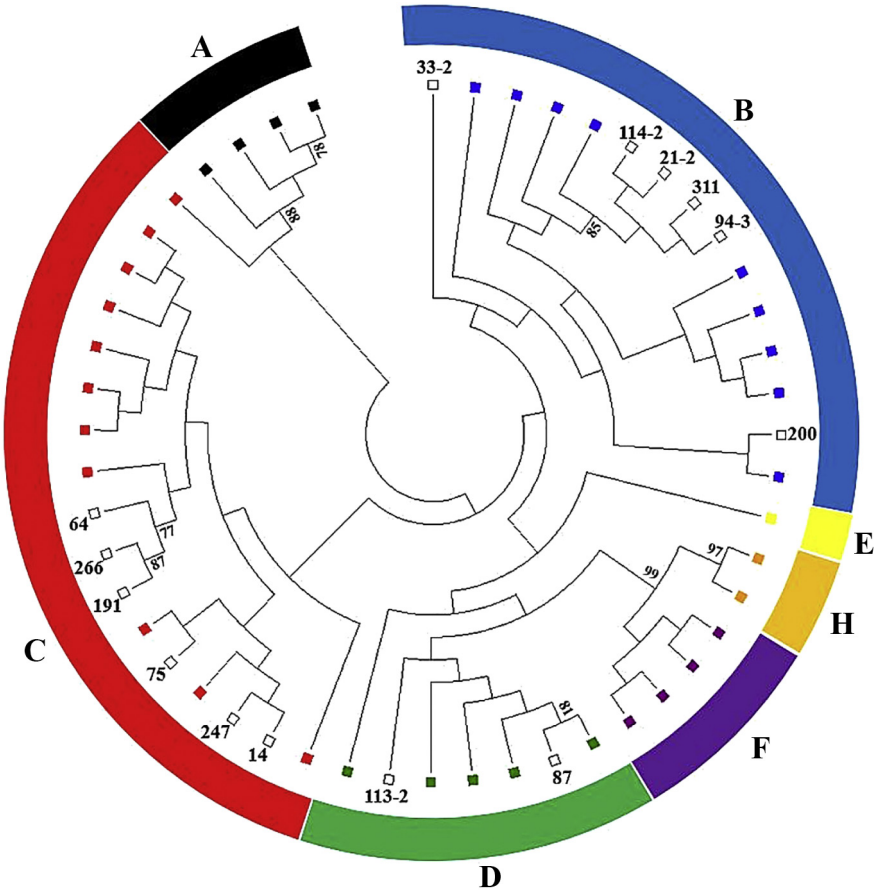

Fig. 2. HBV genotype determination by maximal likelihood constructed phylogenetic analysis of HBV reference sequences (filled square labels) with HBV surface genomic nested PCR amplicons derived from cohort plasma samples (unfilled square labels). Sequences were aligned with MAFFT and trimmed to remove nested PCR primer sequences to a final length of 297 bp. 1000 bootstrap replicates were conducted under the Kimura-2-parameter with MEGA7. Branch nodes with bootstrap values >70 are included next to the corresponding node. Viral genotypes B to D were identified in 12 plasma samples derived from chronic hepatitis B carriers with clinically undetectable viremia. 2 cases (21–2 and 33–2) previously analyzed with INNO-LiPa was similarly identified as genotype B.

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Disclosures and Conflict of Interest

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Ethics ID

The study received approval from the local ethics review board committee at the University of Calgary (i.e., Conjoint Ethics Review Board, Ethics REB-16636).

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