Int J Mol Epidemiol Genet 2017;8(3):19-26
www.ijmeg.org /ISSN:1948-1756/IJMEG0054159

Original Article

Untypable genotype restriction patterns and surface gene variants of hepatitis B virus isolates

Michael O Baclig[1], Karen G Reyes[1], Veni R Liles[1], Juliet Gopez-Cervantes[2]

[1]Research and Biotechnology, St. Luke's Medical Center-Quezon City, Philippines; [2]Liver Disease and Transplant Center, St. Luke's Medical Center-Quezon City, Philippines

Received March 21, 2017; Accepted June 16, 2017; Epub June 20, 2017; Published June 30, 2017

Abstract: Chronic hepatitis B is a global health problem, and is one of the leading causes of cirrhosis and hepatocellular carcinoma. Hepatitis B virus (HBV) genotyping helps in decision making for clinical management of HBV infection, and is important for epidemiological studies. The objectives of this study were to investigate the distribution of HBV genotypes circulating in the Philippines; molecularly characterize untypable genotype restriction patterns; and analyze the presence of surface gene variants. HBV genotypes were determined by restriction fragment length polymorphism (RFLP) and DNA sequencing. Three genotypes, HBV A (76%; 73/96), HBV B (10%; 10/96) and HBV C (14%; 13/96) were detected by RFLP. Out of the 96 isolates, 9% were untypable by RFLP analysis. DNA sequencing followed by phylogenetic analysis revealed that these isolates belonged to HBV genotypes A (67%; 6/9), B (11%; 1/9) and C (22%; 2/9). Out of the 9 isolates, 55% showed single or multiple variations which resulted to amino acid changes. Overall, the identification of untypable genotype can be resolved by sequence and phylogenetic analysis of the S gene and this approach can also be used to detect single or multiple variants. Our findings underscore the importance of accurate genotyping and detection of surface gene variants by DNA sequencing for optimal clinical management.

Keywords: Chronic hepatitis B, genotyping, surface gene variants, untypable

Introduction

Chronic hepatitis B (CHB) remains to be a major health concern affecting nearly 350 million people worldwide. Approximately 1 million deaths occur each year as a result of HBV-related cirrhosis, hepatic decompensation and hepatocellular carcinoma [1-3]. Immunomodulatory agents such as standard interferon α2b or pegylated interferon α2a, and nucleos (t) ide analogues such as lamivudine (LAM, a cytidine L-nucleoside analogue), adefovir dipivoxil (ADV), entecavir (ETV; D-cyclopentane), tenofovir disoproxil fumarate (TDF; an acyclic phosphonate structurally related to adefovir), telbivudine (LdT) and clevudine (CLV) are the antivirals used to treat CHB [4-6].

Hepatitis B virus is a DNA virus that contains approximately 3200 nucleotides in the genome. At least 10 HBV genotypes (A to J) and 4 serotypes (adr, adw, ayr, ayw) have been identified [7, 8]. Genotype A is prevalent in North America, Europe, Southern and Eastern Africa. Genotypes B and C are predominant in Southeast Asia, while genotype D is common in the Mediterranean, India and the Middle East region. Genotype E is localized in Africa, while genotype F has been found in Central and Southern America. Genotype G has been identified in North America, France and Germany. Genotype H has been described in Mexico and Central America. Genotype I have been isolated in Vietnam and Laos, while genotype J was found in Japan [9-13].

There is increasing evidence that HBV genotype influences the clinical course of disease and the response to antiviral therapy. A number of studies have shown that genotype C is associated with more severe liver damage than genotype B. Other studies have shown that genotype A-infected patients are more responsive to antiviral treatment compared to genotype D-infected individuals [2, 7].

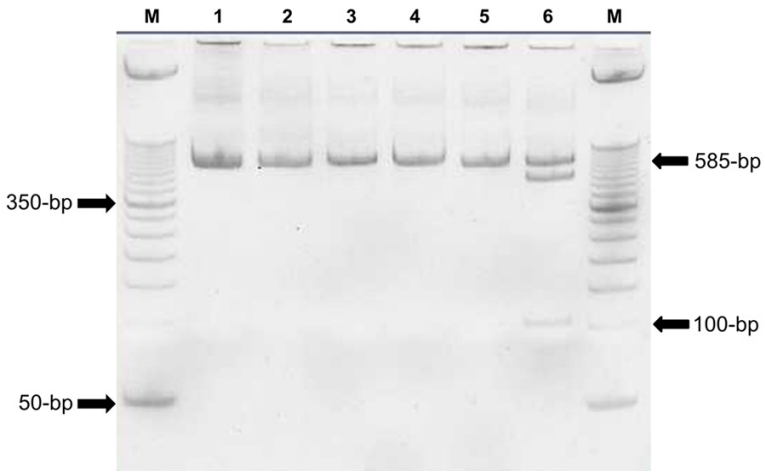

Figure 1. Ethidium bromide-stained 10% polyacrylamide gel showing the undigested and digested amplicons used for HBV genotyping (isolate 11-1470). Lane 1: undigested PCR product of HBV DNA; Lane 2: digested with *Bsr* I (uncut); Lane 3: digested with *Sty* I; Lane 4: digested with *Dpn* I; Lane 5: digested with *Hpa* II; Lane 6: digested with *Eae* I. M: molecular weight marker.

Table 1. HBV genotyping by RFLP and DNA sequencing

Sample Code	RFLP Analysis	DNA Sequencing	GenBank Accession
12-1545	Uncut with *Sty* I	Genotype C	KY171953
12-1544	Uncut with *Bsr* I	Genotype A	KY171954
12-1536	Uncut with *Bsr* I	Genotype B	KY171955
12-1489	Uncut with *Bsr* I	Genotype A	KY171956
12-1485	Uncut with *Bsr* I	Genotype A	KY171957
12-1484	Uncut with *Bsr* I	Genotype A	KY171958
12-1477	Uncut with *Bsr* I	Genotype A	KY171959
11-1470	Uncut with *Bsr* I	Genotype A	KY171960
11-1469	Uncut with *Sty* I	Genotype C	KY171961

Several HBV genotyping platforms have been developed such as restriction fragment length polymorphism (RFLP), enzyme-linked immunosorbent assay, line probe assay, microarray, dot blot, multiplex PCR, real-time PCR and DNA sequencing [14-17]. However, the gold standard for HBV genotyping is DNA sequencing followed by phylogenetic analysis. RFLP is a simple and cost-effective method for HBV genotyping. This technique has been used in studying the influence of viral genotype on the course of disease; assessing response to treatment; and studying the molecular epidemiology of infectious pathogens [15, 18-19].

The objectives of this study were to investigate the distribution of HBV genotypes circulating in the Philippines; molecularly characterize untypable genotype restriction patterns; and analyze the presence of surface gene variants. Direct DNA sequencing was done to confirm the HBV genotypes obtained by RFLP analysis, and to analyze point mutations or multiple variants across the "a" determinant region, which is the most important target for diagnosis and prophylaxis. Understanding the distribution of HBV genotypes and surface gene variants among various ethnic populations could contribute to optimization of treatment, effective vaccination and development of novel molecular diagnostics.

Materials and methods

Samples and patients

Ninety-six anonymized stored blood samples collected from patients positive for HBV DNA by nested PCR from December 2007 to August 2016 at St. Luke's Medical Center were retrospectively analyzed.

Viral DNA extraction, HBV DNA detection and genotyping

The viral DNA was extracted from plasma using the QIAamp DNA Blood Mini Kit according to manufacturer's instructions (Qiagen, Hilden, Germany). HBV DNA amplification was done as previously described [20]. The amplicons were digested with restriction endonucleases such as *Sty* I, *Dpn* I, *Hpa* II and *Eae* I for 16 hours at 37°C and *Bsr* I at 65°C (New England Biolabs). The digested amplicons were analyzed on 10% polyacrylamide gel followed by staining with ethidium bromide and visualized under a UV transilluminator (Bio-Rad Gel Doc, Milan, Italy). A 50-bp ladder (Invitrogen) was used as molecular weight marker (**Figure 1**).

DNA sequencing of HBV

The amplicons in the S gene were purified and sequenced using Big Dye Terminator

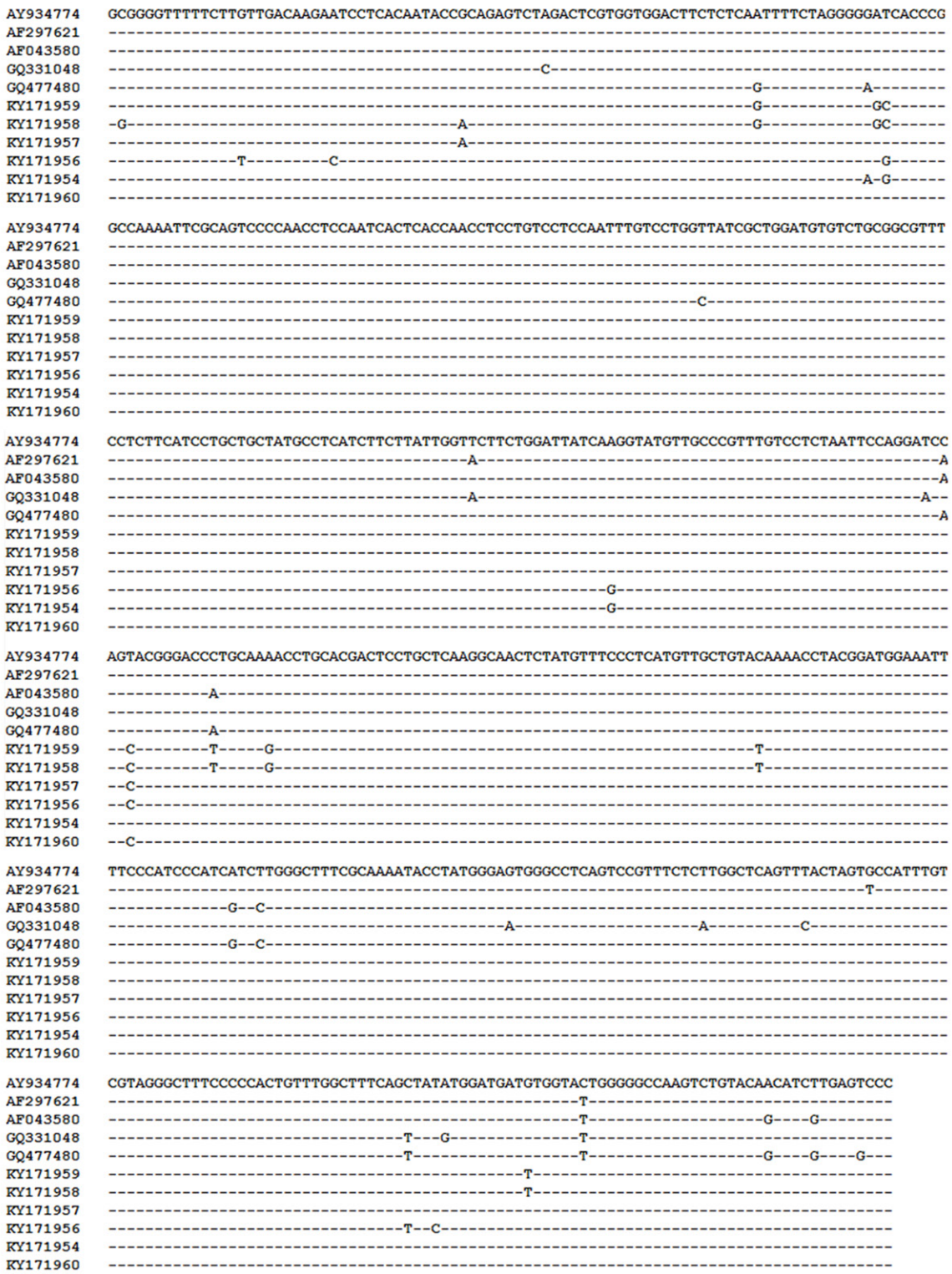

Figure 2. Sequence alignment of S gene of HBV genotype A isolates. At the top, HBV reference sequences are shown (AY934774, AF297621, AF043580, GQ331048, GQ477480). At the left, the isolate identification numbers are shown. The dash indicates the same nucleotide as the first line.

Sequencing Ready Kit and Applied Biosystems 3730xl Automated Sequencer (Macrogen, Korea). The DNA sequences were aligned with the reference sequences of confirmed HBV

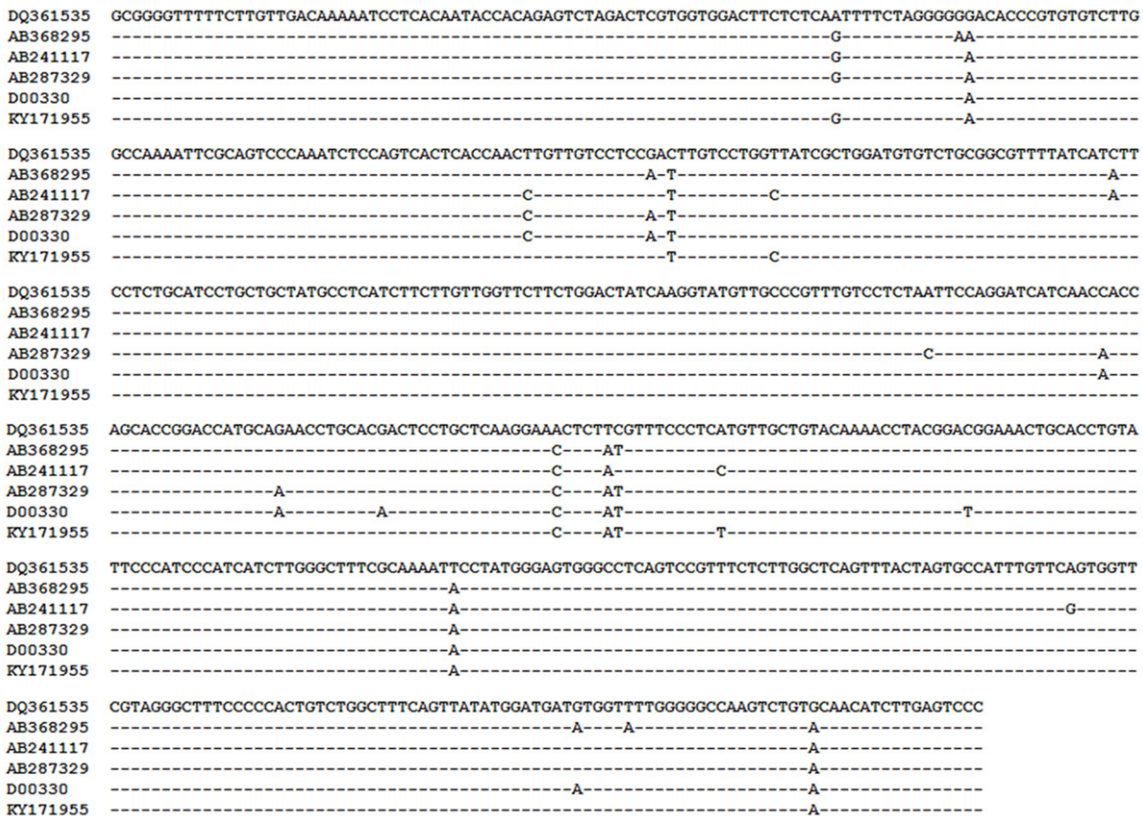

Figure 3. Sequence alignment of S gene of HBV genotype B isolate. At the top, HBV reference sequences are shown (DQ361535, AB368295, AB241117, AB287329, D00330). At the left, the isolate identification number is shown. The dash indicates the same nucleotide as the first line.

genotypes using Clustal W Multiple alignment application contained in BioEdit software version 7.2.5.

Analysis of surface gene variants

The presence of surface gene variants that lie between amino acid positions 100 to 160 were considered point mutations or sequence variations. The nucleotide sequences were aligned with prototype sequences from NCBI GenBank and translated into amino acid sequences.

Phylogenetic analysis

The phylogenetic tree of the S gene was constructed using MEGA software version 6 in accordance with the Neighbor-Joining method. The robustness of the tree was evaluated by 1,000 bootstrap replicates. HBV DNA sequence of a non-human primate was used as an outgroup.

DNA sequence accession numbers

The GenBank accession numbers of HBV used in the analysis were AY934774, AF297621, AF043580, GQ331048, GQ477480, DQ361535, AB368295, AB241117, AB287329, D00330, EU560441, AB033556, DQ478901, GQ377642 and AJ131571. The nucleotide sequences reported in this work can be retrieved under GenBank accession numbers KY171953 to KY171961.

Results

HBV DNA amplification and determination of viral genotypes

HBV DNA was successfully amplified in 96 isolates by nested PCR (100%). Three genotypes, HBV A (76%; 73/96), HBV B (10%; 10/96) and HBV C (14%; 13/96) were detected by RFLP. HBV genotypes D to J were not detected.

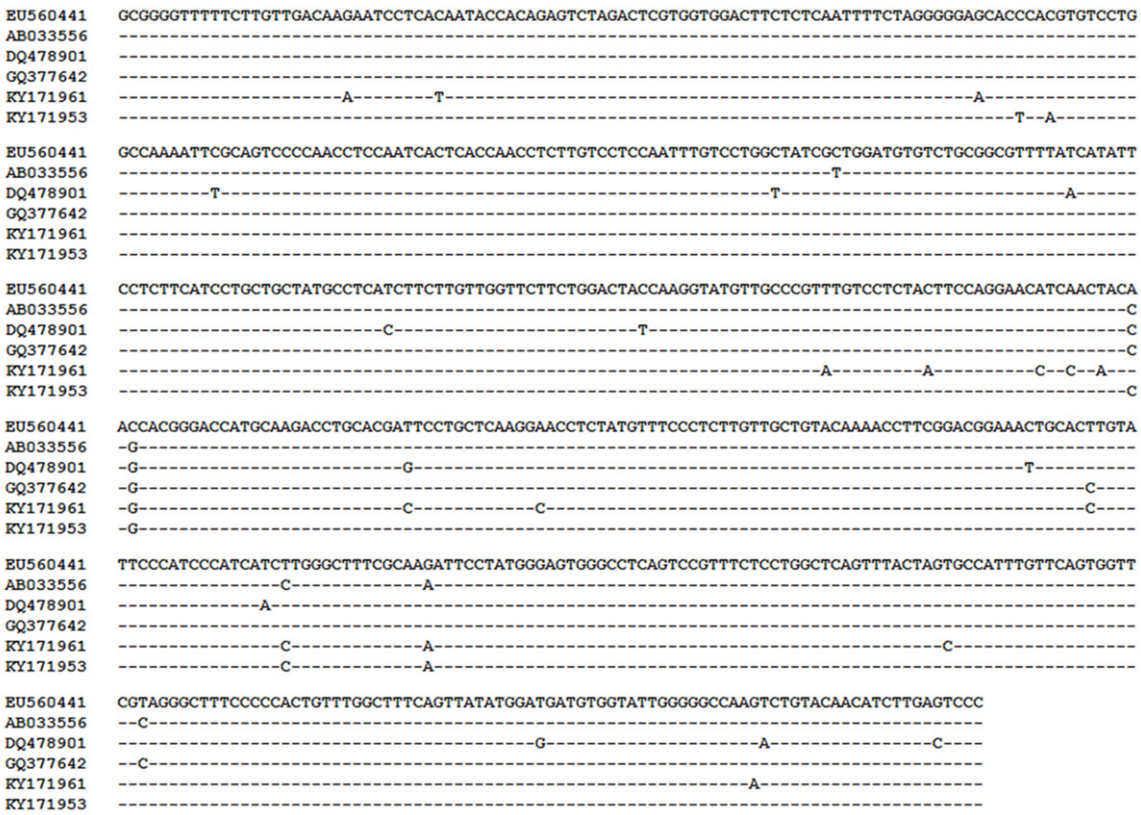

Figure 4. Sequence alignment of S gene of HBV genotype C isolates. At the top, HBV reference sequences are shown (EU560441, AB033556, DQ478901, GQ377642). At the left, the isolate identification numbers are shown. The dash indicates the same nucleotide as the first line.

DNA sequencing and phylogenetic analysis of untypable isolates

Out of the 96 isolates, 9% were untypable by RFLP analysis (**Table 1**). DNA sequencing followed by phylogenetic analysis revealed that these isolates belonged to HBV genotypes A (67%; 6/9), B (11%; 1/9) and C (22%; 2/9). The sequence alignment of the isolates is shown in **Figures 2**-**5**.

Analysis of amino acid variations

Out of the 9 isolates, 55% showed single or multiple variations which resulted to amino acid changes as follows: Leucine (L) to Isoleucine (I) at position 110, Threonine (T) to Serine (S) at position 117, Isoleucine (I) to Threonine (T) at position 126, Asparagine (N) to Threonine (T) at position 131, Serine (S) to Methionine (M) at position 133, Threonine (T) to Isoleucine (I) at position 140 and Arginine (R) to Lysine (K) at position 160 (**Figure 6**).

Discussion

Hepatocellular carcinoma is the fourth leading cancer, and the second leading cause of cancer death in the Philippines. It has been reported that an estimated 7 million Filipino adults or 16% are infected with HBV [21]. To date, 10 HBV genotypes have been identified and they show distinct geographical distribution. In the present study, the most prevalent genotype was HBV A (76%; 73/96). This finding is in agreement with our previous data which showed that genotype A (81%; 43/53) is the most predominant in the country [20].

Clinical outcomes of CHB vary widely. Both host and viral factors have been found to be associated with disease outcomes. A previous study has showed that patients infected with HBV genotypes C or D have lower rates of HBeAg seroconversion as compared with HBV genotypes A or B. In another study, response to antiviral therapy is higher in genotype A-infected

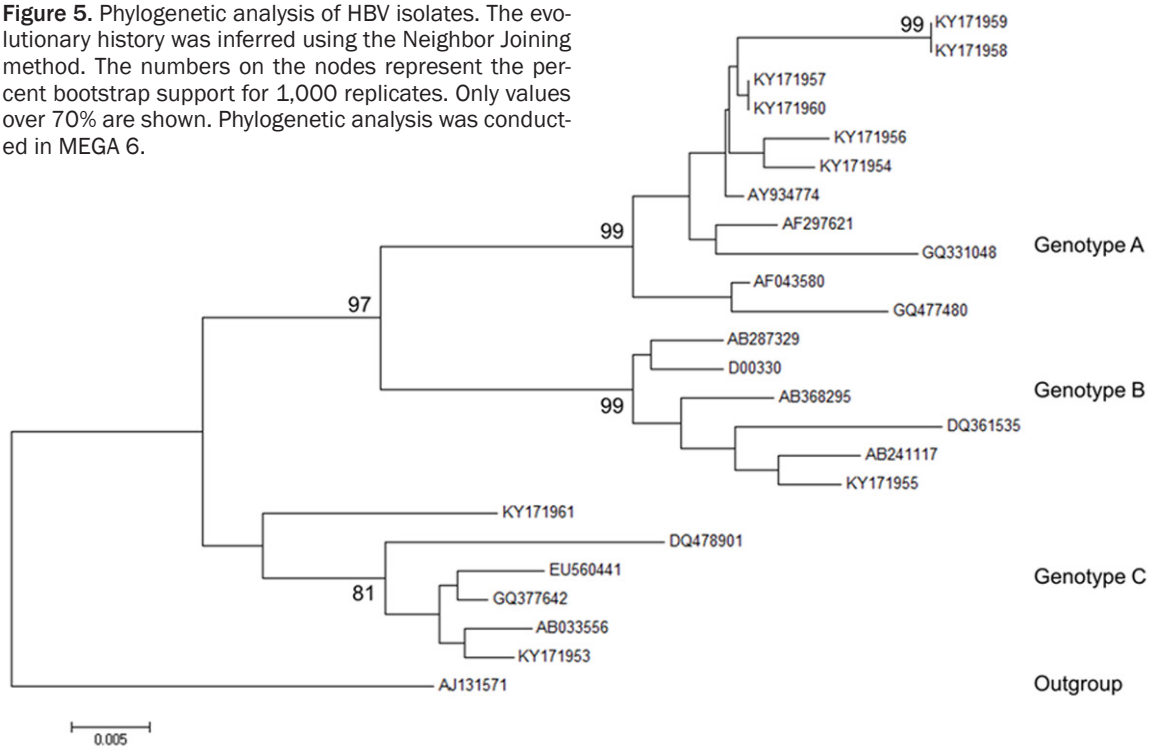

Figure 5. Phylogenetic analysis of HBV isolates. The evolutionary history was inferred using the Neighbor Joining method. The numbers on the nodes represent the percent bootstrap support for 1,000 replicates. Only values over 70% are shown. Phylogenetic analysis was conducted in MEGA 6.

```
Genotype A   100       110       120       130       140       150       160
             |....|....|....|....|....|....|....|....|....|....|....|....|...
AY934774     YQGMLPVCPLIPGSTTTSTGPCKTCTTPAQGNSMFPSCCCTKPTDGNCTCIPIPSSWAFAKYLWEWASV
AF297621     ----------------------------------------------------------------
AF043580     ----------------------------------------------------------------
GQ331048     ----------T-----------------------------------------------------
GQ477480     ----------------------------------------------------------------
KY171959     ----------------------------------------I-----------------------
KY171958     ----------------------------------------I-----------------------
KY171957     ----------------------------------------------------------------
KY171956     ----------------------------------------------------------------
KY171954     ----------------------------------------------------------------
KY171960     ----------------------------------------------------------------

Genotype B   100       110       120       130       140       150       160
             |....|....|....|....|....|....|....|....|....|....|....|....|...
DQ361535     YQGMLPVCPLIPGSSTTSTGPCRTCTTPAQGNSSFPSCCCTKPTDGNCTCIPIPSSWAFAKFLWEWASV
AB368295     ------------------------------T-M---------------------------Y-------
AB241117     ------------------------------T-T---------------------------Y-------
AB287329     ----------L----------K--------T-M---------------------------Y-------
D00330       ----------------------K--------T-M---------------------------Y-------
KY171955     ------------------------------T-M---------------------------Y-------

Genotype C   100       110       120       130       140       150       160
             |....|....|....|....|....|....|....|....|....|....|....|....|...
EU560441     YQGMLPVCPLLPGTSTTTTGPCKTCTIPAQGTSMFPSCCCTKPSDGNCTCIPIPSSWAFARFLWEWASV
AB033556     ----------------S-------------------------------------------K-------
DQ478901     ----------------S--------S-----------------------------T-----------
GQ377642     ----------------S----------------------------------------------------
KY171961     ----------I------S--------T-----------------------------------K-------
KY171953     ----------------S-------------------------------------------K-------
```

Figure 6. Deduced amino acid alignment of S gene of HBV isolates. At the top, HBV reference sequences are given (AY934774, AF297621, AF043580, GQ331048, GQ477480, DQ361535, AB368295, AB241117, AB287329, D00330, EU560441, AB033556, DQ478901 and GQ377642). At the left, the isolate identification numbers are shown. The dash indicates the same amino acid as the first line.

patients than those infected with genotypes B or C. Taken together, these findings suggest pathogenic differences between viral genotypes [22, 23]. Thus, HBV genotyping is important in identifying individuals at risk of disease progression; and genotyping can aid physicians in decision making for optimal clinical management.

RFLP is a widely used technique to identify HBV genotypes. However, genomic variations at enzyme restriction sites in some blood samples may produce atypical restriction pattern or untypable genotype [24, 25]. In the present study, only 91% (87/96) of the HBV isolates produced a fragment characteristic for genotypes A, B or C following restriction digestion with *Sty* I, *Dpn* I, *Hpa* II, *Eae* I and *Bsr* I. The untypable isolates found in this study were resolved by direct DNA sequencing of the S gene. Phylogenetic analysis of the S gene showed that all genotype A isolates clustered together supported by bootstrap value of 99%.

Point mutations, single or multiple variants may affect the antigenicity or immunogenicity of S gene which may lead to false-negative assay and vaccine escape [12, 26]. The well-described methionine (M) to threonine (T) substitution at position 133 (M133T) associated with failure of HBsAg detection and glycine (G) to arginine (R) substitution at position 145 (G145R) associated with immune escape were not found in this study.

Our study comes with some limitations. The subgenotype classification of the isolates cannot be accurately determined by partial sequencing of the S gene. Thus, full-length genome sequencing is recommended in future studies to identify HBV subgenotypes. With the advent of modern and high-throughput technologies such as next-generation sequencing, it is plausible to further characterize HBV isolates to detect novel subgenotypes, unique surface gene variants, minor populations or resistance mutations [27].

Overall, the identification of an untypable genotype can be resolved by sequence and phylogenetic analysis of the S gene and this approach can also be used to detect single or multiple variants. Our findings underscore the importance of accurate genotyping and detection of surface gene variants using DNA sequencing for optimal clinical management.

Acknowledgements

This work was supported by St. Luke's Medical Center through the Research and Biotechnology Group, with the collaboration of the St. Luke's Liver Disease and Transplant Center.

Disclosure of conflict of interest

None.

Address correspondence to: Michael O Baclig, Research and Biotechnology, St. Luke's Medical Center-Quezon City, Philippines. E-mail: mobaclig@stluke.com.ph

References

[1] Kao JH, Chen DS. Global control of hepatitis B virus infection. Lancet Infect Dis 2002; 2: 395-403.

[2] Guirgis BS, Abbas RO, Azzazy HM. Hepatitis B virus genotyping: current methods and clinical implications. Int J Infect Dis 2010; 14: e941-53.

[3] Revill P, Testoni B, Locarnini S, Zoulim F. Global strategies are required to cure and eliminate HBV infection. Nat Rev Gastroenterol Hepatol 2016; 13: 239-248.

[4] Clarke B, Bloor S. Molecular genotyping of hepatitis B virus. J Clin Virol 2002; 25 Suppl 3: S41-45.

[5] Sundaram V, Kowdley K. Management of chronic hepatitis B infection. BMJ 2015; 351: h4263.

[6] Lin CL, Kao JH. Novel therapies for hepatitis B virus cure-advances and perspectives. Aliment Pharmacol Ther 2016; 44: 213-222.

[7] Lin CL, Kao JH. Hepatitis B virus genotypes and variants. Cold Spring Harb Perspect Med 2015; 5: a021436.

[8] Chu CJ, Lok AS. Clinical significance of hepatitis B virus genotypes. Hepatology 2002; 35: 1274-1276.

[9] Hannachi N, Fredj NB, Bahri O, Thibault V, Ferjani A, Gharbi J, Triki H, Boukadida J. Molecular analysis of HBV genotypes and subgenotypes in the central-east region of Tunisia. Virol J 2010; 7: 302.

[10] Ochwoto M, Chauhan R, Gopalakrishnan D, Chen CY, Ng'ang'a Z, Okoth F, Kioko H, Kimotho J, Kaiguri P, Kramvis A. Genotyping and molecular characterization of hepatitis B virus in liver disease patients in Kenya. Infect Genet Evol 2013; 20: 103-110.

[11] Burns GS, Thompson AJ. Viral hepatitis B: clinical and epidemiological characteristics. Cold Spring Harb Perspect Med 2014; 4: a024935.
[12] Al Baqlani SA, Sy BT, Ratsch BA, Al Naamani K, Al Awaidy S, Busaidy SA, Pauli G, Bock CT. Molecular epidemiology and genotyping of hepatitis B virus of HBsAg-positive patients in Oman. PLoS One 2014; 9: e97759.
[13] Malmström S, Berglin-Enquist I, Lindh M. Novel method for genotyping hepatitis B virus on the basis of TaqMan real-time PCR. J Clin Microbiol 2010; 48: 1105-1111.
[14] Lindh M, Gonzalez JE, Norkrans G, Horal P. Genotyping of hepatitis B virus by restriction pattern analysis of a pre-S amplicon. J Virol Methods 1998; 72: 163-174.
[15] Zeng GB, Wen SJ, Wang ZH, Yan L, Sun J, Hou JL. A novel hepatitis B virus genotyping system by using restriction fragment length polymorphism patterns of S gene amplicons. World J Gastroenterol 2004; 10: 3132-3136.
[16] Osiowy C, Giles E. Evaluation of the INNO-LiPA HBV genotyping assay for determination of hepatitis B virus genotype. J Clin Microbiol 2003; 41: 5473-5477.
[17] Ali MM, Hasan F, Ahmad S, Al-Nakib W. Comparative evaluation of INNO-LiPA HBV assay, direct DNA sequencing and subtractive PCR-RFLP for genotyping of clinical HBV isolates. Virol J 2010; 7: 111.
[18] Ouneissa R, Bahri O, Ben Yahia A, Touzi H, Azouz MM, Ben Mami N, Triki H. Evaluation of PCR-RFLP in the pre-S region as molecular method for hepatitis B virus genotyping. Hepat Mon 2013; 13: e11781.
[19] Rendón JC, Cortés-Mancera F, Duque-Jaramillo A, Ospina MC, Navas MC. Analysis of hepatitis B virus genotypes by restriction fragment length polymorphism. Biomedica 2015; 36: 79-88.
[20] Baclig MO, Alvarez MR, Gopez-Cervantes J, Natividad FF. Unique surface gene variants of hepatitis B virus isolated from patients in the Philippines. J Med Virol 2014; 86: 209-216.
[21] Wong SN, Ong JP, Labio ME, Cabahug OT, Daez ML, Valdellon EV, Sollano JD Jr, Arguillas MO. Hepatitis B infection among adults in the Philippines: a national seroprevalence study. World J Hepatol 2013; 5: 214-219.
[22] Brunetto MR, Marcellin P, Cherubini B, Yurdaydin C, Farci P, Hadziyannis SJ, Rothe V, Regep L, Bonino F. Response to peginterferon alfa-2a (40KD) in HBeAg-negative CHB: on-treatment kinetics of HBsAg serum levels vary by HBV genotype. J Hepatol 2013; 59: 1153-1159.
[23] Liu CJ, Kao JH. Global perspective on the natural history of chronic hepatitis B: role of hepatitis B virus genotypes A to J. Semin Liver Dis 2013; 33: 97-102.
[24] Amini-Bavil-Olyaee S, Tacke F, Alavian SM. HBV Subgenotypes D1, D2, D-del! Are old genotyping methods interpreted correctly? Hepat Mon 2013; 13: e13048.
[25] Pourkarim MR, Amini-Bavil-Olyaee S, Lemey P, Maes P, Van Ranst M. Are hepatitis B virus "subgenotypes" defined accurately? J Clin Virol 2010; 47: 356-360.
[26] Abdelnabi Z, Saleh N, Baraghithi S, Glebe D, Azzeh M. Subgenotypes and mutations in the S and polymerase genes of hepatitis B virus carriers in the West Bank, Palestine. PLoS One 2014; 9: e113821.
[27] Lowe CF, Merrick L, Harrigan PR, Mazzulli T, Sherlock CH, Ritchie G. Implementation of next-generation sequencing for hepatitis B virus resistance testing and genotyping in a clinical microbiology laboratory. J Clin Microbiol 2016; 54: 127-33.