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Establishment of reference sequences of hepatitis B virus genotype B subgenotypes

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Abstract: Hepatitis B virus (HBV) has been classified into ten genotypes (A-J). Genotype B (HBV/B) is divided into nine subgenotypes (B1-B9), each with specific geographical predominance. Some reference sequence of HBV/B subgenotypes are currently in use, but these sequences have defects, being insufficient to represent the reference of individual subgenotype. The aim of this study was to establish a more representative reference of HBV/B subgenotypes in different regions. Full genomic sequences of HBV/B were obtained from GenBank and compartmentalized into genomic subtypes. The homology between our established HBV/B subgenotype references was evaluated at the nucleotide level. We established reference strains for B1-Japan, B2-China, B3-Indonesia, B4-Vietnam, B6-North America, B7-Indonesia and B9-Southeast Asia. Fractional significant mutation sites of the strains that were established were observed in the BCP/Pre-C regions. We calculated the genetic divergence time from the most recent common ancestor of HBV/B pedigree. The reference sequences established in the study provide reference standards for studies on molecular characterization, virology and pathogenesis of HBV/B.

Keywords: hepatitis B virus; genotype; phylogenetic analysis; reference strains; bioinformatics

INTRODUCTION

Hepatitis B virus (HBV) is an important global public health problem that has infected approximately 2 billion people worldwide and has more than 240 million chronic carriers [1,2]. About 6 million people die annually from primary adverse outcomes such as liver failure, cirrhosis and hepatocellular carcinoma (HCC) [3]. The clinical course in infected HBV individuals depends on complex interactions among various factors, including viruses, hosts, environments, genotypes, mutations and subgenotypes [4]. To date, the HBV genome has been phylogenetically classified into ten distinct genotypes (A-J) in accordance with the gene sequence [5,6] based on greater than 8% nucleotide variation in the genome and 4-8% nucleotide differences in the subgenotype [7].

Among all the HBV genotypes, HBV genotype B and C are the most common and predominant genotype, the reference sequence of genotype C subgenotypes has been established [8], but the reference sequence of HBV genotype B (HBV/B) subgenotypes which is more representative than the previously existing sequence of HBV/B subgenotypes is still scarce. There is a great need to establish a new reference sequence of HBV/B subgenotypes. The subgenotypes of HBV/B are denoted by numerical subscripts (B1, B2, B3, etc.), and each subgenotype has different geographical predominance. Subgenotype B1 is the original Bj subgenotype, which is predominant in Japan [9]. B2 is a predominant subgenotype in Asia which has been seen in China, Malaysia, Taiwan and Thailand. B3 has been reported in Indonesia, whereas B4 is relatively rare and has been reported in Vietnam [10]. The subgenotype B5 is common in the Philippines [11], while B6 is a new genotype recently found in the Arctic region in indigenous populations from the USA, Canada, and Greenland. B7 and B8 have been reported in Indonesia, and B9 is a recently reported subgenotype observed in Indonesia [10].

The different HBV/B subgenotypes may virologically differ in their capacity to induce clinical disease [13]. In addition, these subgenotypes have distinct geographical distributions and may influence the HBV biology and clinical diseases in hosts. Therefore, the reference sequences of HBV/B subgenotypes are significant for sequence study. Some reference sequences of HBV/B subgenotypes now have been used [14]; however, these reference strains were either established with a limited number of isolates or determined with the first isolated strain and were not sufficient to represent the reference of individual subgenotype. It is necessary to establish a representative reference sequence of the HBV/B subgenotype, which may contribute to the experimental primer design of HBV. The established reference sequence was obtained by comparing the genotype individually with an unprecedented large number of HBV strains and were more representative. In this study, the whole genome sequence of HBV/B genotypes in different regions was obtained from . The reference sequence of HBV/B subgenotypes was established, and the gene sequences also have been compared to understand the various sites of genetic variation. These established reference sequences can be used to design synthetic HBV/B viruses, and they can also provide appropriate standards for studies on the epidemiological and virological features of HBV/B.

MATERIALS AND METHODS

Collection of sequences

A total of 3435 complete HBV genome sequences were previously retrieved from the GenBank database using the phylogenetic approach in December 2015 [15]. Search terms included HBV, genotype, and complete genome. The subgenotypes of HBV/B isolates were combined by phylogenetic analysis using the CLUSTAL W method in MEGA 6 software [16]. The following sequences were excluded: (i) sequences with a number of nucleotides bases greater than 3300 or less than 3100, because the HBV/B complete genome sequences consist of 3215 nucleotides; (ii) sequences with insufficient number of nucleotides, such as B5 and B8; and (iii) sequences with recombinant strains, including B/C, B/D, B/E and B/G. Finally, 523 full-length sequences of HBV/B subgenotypes were selected. Phylogenetic analysis results were further confirmed by visual inspection.

Reference sequences

Reference sequences of each HBV/B subgenotype in distinct regions were established with each nucleotide of the reference sequence, which considers the maximum nucleotide compatibility in the same site for all sequences. Dots with different colors indicated the rate of base alternation. Black dots indicated that the position had no alteration; blue dots indicated that the position had greater than 5% alteration; red dots indicated that the position had greater than 20% alteration; and N in the positions showed multiple base alterations. Each nucleotide position was regarded as the greatest frequency of a base as the reference nucleotide, obtaining the final reference sequences of HBV/B subgenotypes. More than 20% of the substituted bases were analyzed, and the base composition of the sites was determined after acquiring the HBV/B subgenotypes and the reference sequences of the HBV/B subgenotypes. Moreover, important partial mutation sites of the nucleotide substitutions in the basal core promoter or the pre-core regions of each HBV/B subgenotype in different regions, such as A1762T/G1764A, A1814/T1858, and G1896A, were inspected.

Phylogenetic analysis

The whole genome sequences of the HBV/B strains based on the GenBank database were arranged in a straight line by the MEGA 6 software program and confirmed by visual inspection [17]. Phylogenetic trees were established using the neighbor-joining method, and the distances were calculated by using the Kimura two-parameter method [15]. Bootstrapping was performed through using 1000 replicates with the confidence of the tree. The genetic divergence time was calculated from the most recent common ancestor based on the nucleotide substitution rate of about 2.2×10-6 per site per year of the full-length sequence [18]. The HBV/B subgenotypes were assigned according to recently reported classification system.

Amino acid and nucleotide analysis of the reference sequences

Homology analysis of the HBV/B subgenotype reference strains was conducted and compared with other reference strains of international nucleotide sequences

using DNAMAN V6 software. The HBV/B subgenotype reference strains were divided into full-length gene sequence comparisons and S gene sequences. The established reference sequences were comprehensively analyzed using previously reported international nucleotide sequences, which were retrieved from the following GenBank reference sequences: X02763 (HBV/A), AF297621 (HBV/A), D00330 (HBV/B), AB073858 (HBV/B), AB033556 (HBV/C), AB048704 (HBV/C), X02496 (HBV/D), X75657 (HBV/E), X69798 (HBV/F), AF160501 (HBV/G), and AY090454 (HBV/H) [5,19]. In addition, homology analysis of the established reference sequences was conducted among the established HBV/B subgenotype reference sequences at the nucleotide level using DNAMAN V6 software. The reference sequences were compared with the full-length gene sequences and the S gene sequences of the established HBV/B reference sequences.

RESULTS

Geographical distribution of the HBV/B subgenotypes

A total of 523 HBV/B whole-length strains were used to establish reference strains. Notably, the seven subgenotypes of HBV/B had different geographical distributions. Twenty-four strains constituted the sequence of HBV/B1 and was reported in 87.50% of HBV/B cases in Japan. There were 385 B2 isolates, which represented the predominant constituents of HBV/B isolates that were widely reported in Asia, including China, Malaysia and other countries. B3 included 25 isolates which comprises 60% of HBV/B in Indonesia. HBV/B4 had 20 strains that were predominantly reported in Vietnam. HBV/B6 included 24 strains, which were widespread in North America. HBV/B7 was reported in Indonesia, with 13 isolates found. HBV/B9 was predominantly reported in Southeast Asia, with 19 isolates found. In these regions, HBV/B1 (Japan), HBV/B2 (China) and HBV/B6 (North America) have been reported as the main local subgenotypes (Table 1). The HBV/B subgenotypes B5 and B8 were excluded because subgenotype B5 had only five full-length strains and subgenotype B8 had only seven full-length isolates.

Table 1. Regional distribution of HBV/B subgenotypes.

Subgenotype	Location	Number	Percentage (%)	Total
B1	Japan	21	87.50%	24
	Others	3	12.50%	
B2	China	285	74.03%	385
	Malaysia	36	9.35%	
	Taiwan	21	5.45%	
	Thailand	13	3.38%	
	Others	30	6.59%	
B3	Indonesia	15	60.00%	25
	Others	10	40.00%	
B4	Vietnam	15	75.00%	20
	Others	5	25.00%	
B6	North America	36	92.31%	39
	Others	3	7.69%	
B7	Indonesia	13	100.00%	13
B9	Southeast Asia	19	100.00%	19

B1: HBV/B subgenotype B1; B2: HBV/B subgenotype B2; B3: HBV/B subgenotype B3; B4: HBV/B subgenotype B4; B6: HBV/B subgenotype B6; B7: HBV/B subgenotype B7; B9: HBV/B subgenotype B9.

Establishment and nucleotide substitution of the HBV/B subgenotypes reference sequences

The reference sequences of the HBV/B subgenotypes, B1, B2, B3, B4, B6, B7 and B9 in different regions were established and were submitted to GenBank under the accession numbers KP341007-KP341013, respectively (Supplementary Fig. S1). In addition, the same subgenotype B2 from different regions was also established in this study (Supplementary Fig. S2). The occurrence of HBV/B subgenotype nucleotide changes at position A1762T/G1764A of the core promoter gene and was significantly different. B2 had the highest mutation rate (118/285, 41.40%; 121/285, 42.46%) at the A1762T/G1764A position, followed by B4, B1, B3 and B9. However, there were no nucleotide substitutions in HBV/B6 (0/36) and HBV/B7 (0/13). In all of the HBV/B strains, the nucleotide position A1814/T1858 showed no mutations. The mutation of G1896A was mainly observed in B2, B3, and B9, while A1896G was primarily observed in B1 and B9, which may be attributed to the limited number of sequences (Fig. 1).

Phylogenetic trees of the HBV/B complete genomes

Phylogenetic trees were constructed by the neighbor-joining algorithm through MEGA software version 6.0. The reference strains and some selected sequences

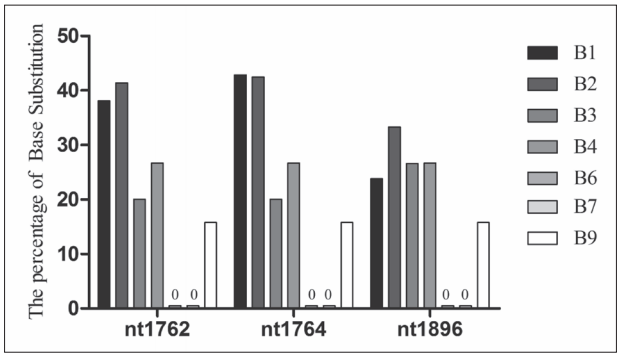

Fig. 1. Important sites of base substitution of HBV/B subgenotypes at different positions. HBV/B2 has the highest mutation rate at position A1762T (41.4%) and G1764A (42.46%), followed by B4, B1, B3 and B9. HBV/B6 and HBV/B7 have no nucleotide substitutions at any nucleotide positions. Mutation G1896A is mainly observed in B2, B3, and B9, and mutation A1896G is primarily observed in B1 and B9.

were clustered by the neighbor-joining method to verify the established reference strains which were classified with the correct strains (Fig. 2). The previously established HBV/Ba strains situated in cluster of B2 reference sequences exhibited more similar clustering with them. The reliability of the inferred phylogenetic trees was assessed by the bootstrap resampling test, which included 1,000 replications. Furthermore, the phylogenetic trees were used to estimate the differentiation time of HBV/B (Fig. 3).

Among the B subgenotypes, subgenotype B6 was determined to have the longest genetic divergence time and diverged from common ancestor about 8.5×103 years ago. The subgenotypes B3, B7 and B9 were determined to have similar genetic divergence times, likely because they are found in Asia. In addition, B1, B2 and B4 were determined to have similar genetic divergence times, likely because they are found in Indonesia. B1, B2 and B4 diverged approximately 4000-7000 years ago, while B3, B7 and B9 diverged 3000 years ago, likely because they have similar genetic divergence times. The newly established HBV/B2 reference strains were determined to have the same genetic divergence times

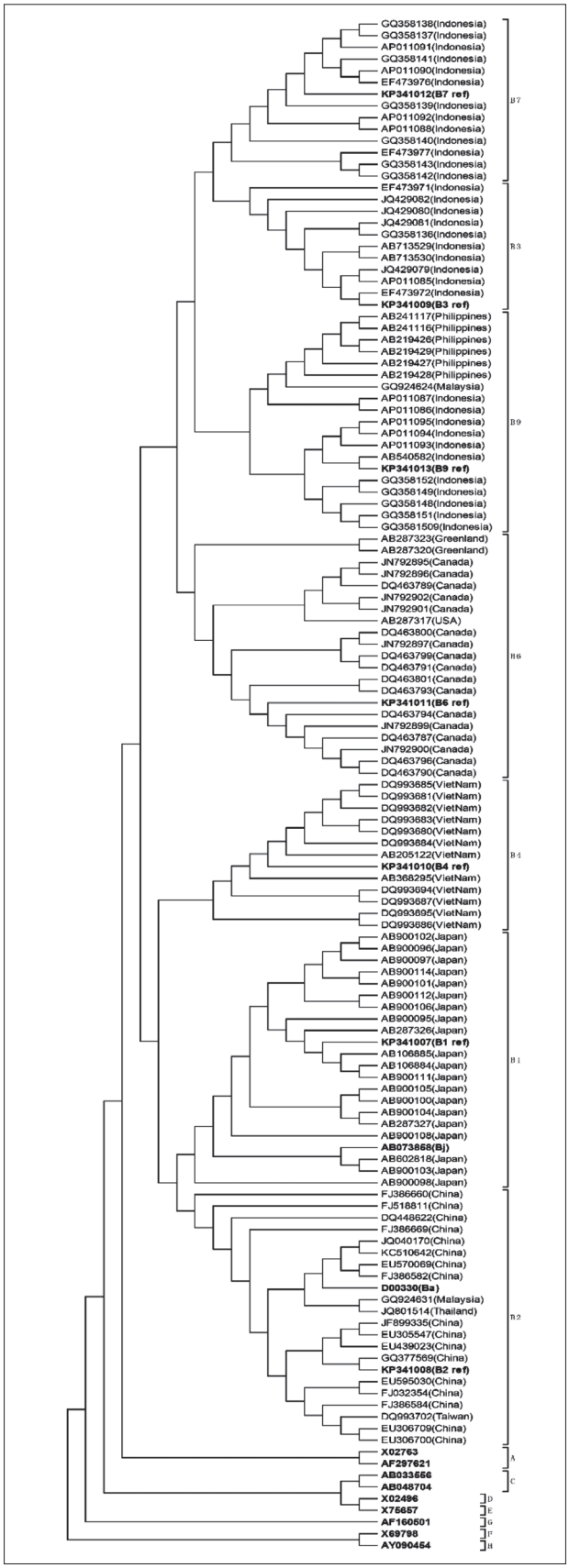

Fig. 2. Phylogenetic trees of reference sequences and some selected strains constructed by the neighbor-joining method. Bootstrap values (1000 replicates) higher than 70% are indicated for the major nodes. Accession numbers and the locations are shown in each branch, and seven reference strains and another eleven whole genome sequences are highlighted in bold. HBV subgenotypes are listed on the right of each respective cluster.

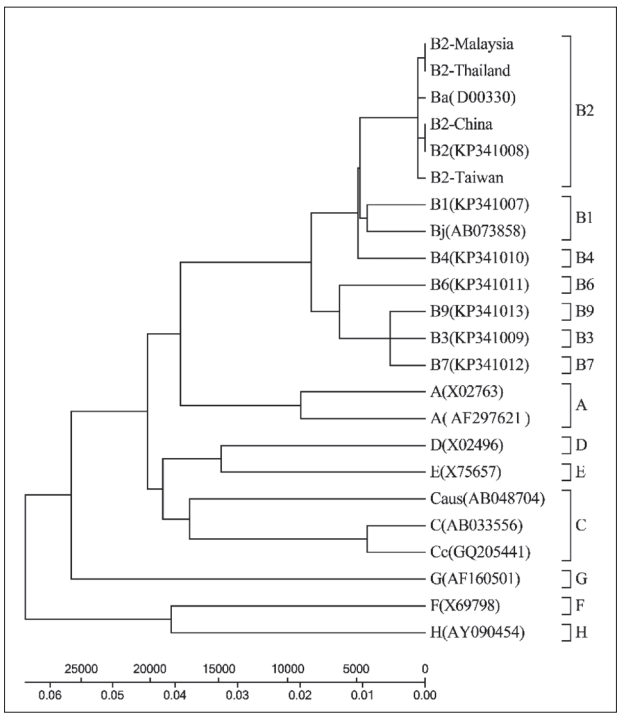

Fig. 3. Phylogenetic trees of the established reference strains of HBV/B subgenotypes and the reference strains of HBV A to H genotypes. The reference strains were established by the neighbor-joining method and calibrated with a substitution rate of 2.2×10-6 per site per year. The branch lengths correspond to the length of time (see the time scale bar at the bottom of the tree). Subgenotypes with corresponding positions are shown in each branch.

as the Ba subgenotypes. Subgenotype B1 was also determined to have the same genetic divergence time as the Bj subgenotypes. B2-Malaysia and B2-Thailand were determined to have the same genetic divergence time and diverged about 150 years ago. Moreover, among the A-H genotypes, the genetic divergence time of genotype A is most similar to the B subgenotypes (Fig. 3).

Nucleotide sequence analysis of reference isolates was conducted by comparing the homology of the established B reference sequences, and it confirmed a sequence divergence >8% over the entire genome, and sequence divergences >4% in the S genes. By comparison, subgenotype B1 mainly showed a greater homology with B2 than with other B sequences, and B2 showed a greater homology with B4. However, subgenotype B3 mainly had a greater homology with B9 and B7 at the nucleotide level than with another B subgenotypes (Table 2).

DISCUSSION

The reference sequences of HBV that can adequately represent strains isolated in a particular epidemic are critical to sequence studies, especially in mutations. The authors of a recent study reported that they proposed a method to establish the consensus sequences of HBV/B. However, they have not constructed the reference sequences of HBV/B subgenotypes [19]. Some reference sequences of HBV/B subgenotypes are currently in use such as Ba and Bj, but these sequences have defects which they are only established from a limited number of strains or determined from the first isolated strain [14]. Therefore, there is an urgent need to consider these defects to establish the reference sequences of HBV/B subgenotypes that are more representative. In this study, compared with the sequences of previous reference strains of HBV/B subgenotypes such as Ba and Bj, the newly established reference sequences displayed greater reliability. The HBV Bj reference sequence was only found in Japan and did not has global presence. The Ba reference sequence was mainly found in Asia, and it also exhibited recombination with genotype C. Phylogenetic analysis confirmed that our newly established reference sequences were clustered with previously reported strains, and the reference sequences that we established belonged to the correct subgenotypes.

HBV genotypes and subgenotypes have distinct geographical distributions and have been shown to differ with regard to the clinical outcome, prognosis, and response to interferon treatment [20-22]. Therefore, it is important to understand

Table 2. Homology analyses of the full genome and S gene among reference strains of established HBV/B subgenotypes.

Reference strains	B1		B2		B3		B4		B6		B7	
	Full	S	Full	S	Full	S	Full	S	Full	S	Full	S
B2	97.0	99.1	-	-								
B3	95.3	98.8	96.6	99.4	-	-						
B4	96.8	98.4	97.8	98.4	96.9	98.7	-	-				
B6	95.7	98.7	94.6	98.7	95.7	98.7	94.7	97.9	-	-		
B7	95.0	98.2	96.2	98.8	98.9	99.4	96.6	98.4	95.7	98.4	-	-
B9	95.8	98.5	97.1	99.1	98.9	99.7	97.3	98.7	96.0	98.4	98.7	99.4

Full indicates complete genome, S indicates S genome.

the roles of different genotypes. HBV/B subgenotypes were selected based on geography, including B1-Japan, B2-China, B3-Indonesia, B4-Vietnam, B6-North America, B7-Indonesia and B9-Southeast Asia. *In vitro* chemical synthesis was not only used to synthesize genome sequences with replication by utilizing a reference sequence but is also practical for experimental research. For example, this method has been exploited in research on the H1N1 influenza virus *in vitro* and *in vivo*. Studies have demonstrated that the genome sequence maintained the corresponding biological functions of the H1N1 influenza virus, although it was newly synthesized and might not exist in nature [23].

Similar studies have also been conducted to investigate the tobacco mosaic virus and the chemically synthesized HBV consensus genome *in vitro* and *in vivo*, which maintained the corresponding biological functions. The results from using these strategies indicated that the established reference sequences were feasible and credible, and that the consensus genome of the HBV genotype B could be used for future studies. Compared with genotype C, HBV genotype B has been associated with a greater rate of IFN-induced HBeAg clearance, an increased sustained treatment response and a higher mean HBV DNA reduction, which may be related to high mutation frequency in the BCP/Pre-C regions [24,25]. Many studies have reported that the presence of BCP (A1762T/G1764A) mutations is associated with an increased risk of severe liver diseases, including HCC, and can be used as an independent predicator for the progression of HCC [26,27]. A commonly reported mutation in the pre-core region is the G1896A mutation, which can cause hepatitis B e antigen (HBeAg) negativity and create a premature stop codon. Compared with patients with genotype C, patients with HBV/B infection have an increased spontaneous clearance rate of HBeAg, decreased levels of viral replication, increased occurrence of acute hepatitis, increased response rate to α-interferon therapy, and decreased incidence of occult HBV infection [28,29]. In our study, the occurrence of HBV/B subgenotype nucleotide changes at position A1762T/G1764A of the core promoter gene was significantly different; B2 had the greatest mutation rate at position A1762T/G1764A, followed by B4, B1, B3 and B9. The mutation of G1896A was mainly observed in B2, B3, and B9. However, these nucleotide substitutions were not found in the HBV subgenotypes B6 and B7. In all of the HBV/B strains, the nucleotide position A1814/T1858 was without mutations. HBV genotypes and subgenotypes have been associated with different virological and clinical characteristics, indicating that they may play a crucial role in the virus-host relationship [30,31]. These results indicated that there were substantial differences among the HBV genotypes and subgenotypes, and these differences can be used in clinical assessments to identify patients at greater risk for disease progression and severity of liver disease. For example, although HBV genotype C causes more severe liver disease than HBV/B in Asia, remarkably, patients infected with HBV/B in Taiwan develop HCC at a much earlier age than those with HBV genotype C, while young Japanese patients infected with HBV/B do not develop HCC [25].

With distinct geographical distributions, HBV/B subgenotypes have different genetic divergence times from the most recent common ancestor based on the nucleotide substitution rate of 2.2×10-6 per site per year [17]. Among the seven reference sequences, subgenotype B6 has the longest genetic divergence time, which indicates that subgenotype B6 may be the original subgenotype. The subgenotypes B3, B7 and B9 have similar genetic divergence times, likely because they are found in Indonesia. Moreover, HBV B1, B2 and B4 have similar genetic divergence times, likely because they are mainly found in Southeast Asia. Thus, we can learn more about the epidemiology of HBV/B, and speculating the route of HBV/B transmission may help to prevent future spread of HBV infection.

CONCLUSION

The reference sequences of HBV/B subgenotypes were established based on the HBV genome sequence data of numerous isolates from different epidemic regions. These sequences were more representative than previously existing sequence of HBV/B subgenotypes, which was proven by phylogenetic and homology analyses. The newly established reference sequences offer appropriate reference standards for studies on the molecular characterization, virology and pathogenesis of HBV/B, including understanding of the various sites of genetic variation. They could also provide suitable standards for the design of primers and further studies on the epidemiology with specific subtypes of HBV/B

in different geographical regions. However, there were several limitations to this study. The clinical information of patients regarding the origin of the virus strain could not be traced, such as the disease stage of HBV patients and the antiviral treatment course, which could affect gene sequence mutations. Some strains might not be retrieved based on the search terms used, and the sequence of some strains come from a limited number of samples. The accuracy of the obtained whole sequences was not completely ensured since they were collected from database. Therefore, future studies on HBV reference sequences are necessary.

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Supplementary Material

The Supplementary Material is available at: http://serbiosoc.org.rs/NewUploads/Uploads/Jin%20et%20al_5724_Supplementary%20Material.pdf