Discrepant Results of Hepatitis B Virus Genotype Determination by PCR and DNA Sequencing

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Abstract

Currently, multiplex-PCR with genotype-specific primers is widely used for preliminary screening of hepatitis B virus (HBV) genotyping, despite its relatively lower accuracy compared with whole genome sequencing. Here, we present the discrepant results of HBV genotyping by PCR and full-length sequencing. HBV DNA was isolated from chronic hepatitis B serum and the HBV genotype was detected by PCR using genotype-specific primers and full-length genome sequencing. As a result, the determination of genotype B by the PCR method was consistent with the DNA sequencing results; however, PCR revealed that genotype C exhibited a mixed genotype of B and C in the current study. In conclusion, the PCR-based genotyping method may not provide accurate information of the HBV genotype and whole genome sequencing remains the “gold standard” method for HBV genotyping.

1. Introduction

Currently, over 290 million people worldwide suffer from chronic hepatitis B virus (HBV) infection (WHO 2021). In China, approximately 7.18% of the population are HBV carriers who are at risk of developing end stages of liver disease, including liver cirrhosis and hepatocellular carcinoma (Chen et al. 2006; Wang et al. 2014).

HBV contains a partially double-stranded DNA within a nucleocapsid surrounded by envelope proteins (Delius et al. 1983). HBV genome replication relies on a viral polymerase that harbors RNA-dependent reverse transcriptase (Summers and Mason 1982; Hu and Seeger 2015). However, the high error rate of RT coupled with a substantial amount of daily production (approximately 10^{11} copies/day), as well as selected immune pressures, frequently results in substantial genetic diversity that may evolve into several HBV genotypes (Lau and Wright 1993; Pourkarim et al. 2014; Lin and Kao 2015).

Recently, HBV has been classified into nine genotypes (A–I) and one tentative genotype J based on more than 7.5% differences in the full-length HBV genome sequences (Kramvis and Kew 2007; McNaughton et al. 2020). In addition, each genotype is further divided into subgenotypes based on more than 4.5% and less than 7.5% nucleotide differences (Kramvis and Kew 2007).

It has been shown that the different HBV genotypes display distinct tendencies of chronic infection, HBeAg seroconversion, and responses to interferon-based therapy (Orito et al. 2001; Kao 2002; Kao et al. 2004; Livingston et al. 2007; Ito et al. 2014; McMahon et al. 2021). Although HBV genotyping has not been widely used in clinical settings, the recent European Association for the Study of the Liver guidelines for HBV treatment recommend different timelines for treatment cessation due to a lack of response for genotypes A–D (European Association for the Study of the Liver. Electronic address and European Association for the Study of the 2017). This indicates that the HBV genotype is beginning to be considered in the context of patient management.

Several molecular-based methods have been developed for HBV genotyping, including polymerase chain reaction (PCR) with genotype-specific primers, PCR-restriction fragment length polymorphism, PCR-invader assay, oligonucleotide microarray chip, and sequencing analysis of the whole or portions of the HBV

genome(Naito et al. 2001; Kirschberg et al. 2004; Chen et al. 2007). Among them, the sequencing analysis of whole HBV genome is considered to be the “gold standard” method for genotyping; however, it is relatively time-consuming and expensive, especially with respect to the large number of samples(Kramvis and Kew 2007; McNaughton et al. 2020). By contrast, since PCR-based genotyping methods are rapid and inexpensive, they are widely used in HBV genotyping; however, any nucleotide substitution in the PCR primer binding site may alter the HBV genotyping results.

In the present study, we found discrepancies in the results between PCR-based methods and the whole HBV genome sequencing analysis. The results and the possible reason for the discrepancy is described.

2. Materials And Methods

2.1 Isolation of serum HBV DNA and PCR-based HBV genotyping

Serum samples from 52 Chronic hepatitis B patients were randomly collected from the first affiliated hospital and the second affiliated hospital of Hainan Medical University, Haikou, Hainan, China. HBV DNA was isolated from 200 µL of serum using a QIAamp MinElute Virus Spin Kit (Qiagen, Cat#5704) in accordance with the manufacturer’s instructions. The sampling procedures were approved by the Ethics Committee of Hainan Medical University.

HBV genotype-specific primers introduced by Naito H et al. were used to detect the HBV genotypes using nested PCR(Naito et al. 2001). In brief, the nucleotide positions from 2,823 to 704 of the HBV genome were first amplified by universal primers using serum HBV DNA as a templates. Next, a second round of PCR was carried out using the mixture of genotype-specific primers (genotypes A, B, and C) (Table 1). The HBV genotype was evaluated based on the migration of PCR products via DNA electrophoresis on a 3% agarose gel. Negative controls were included at each step of the PCR reaction to avoid false-positive results.

Table 1
List of PCR primer pairs

	Primers	Sequence (5'→3')	Nucleotide position
Genotypic PCR			
First round	P1	5'-TCACCATATTCTTGGGAACAAGA-3'	nt 2823-2845
	S1-2	5'-CGAACCACTGAACAAATGGC-3'	nt 685-704
Second round	genotype A	5'-CTCGCGGAGATTGACGAGATGT-3'	nt 113-134
	genotype B	5'-CAGGTTGGTGAGTGACTGGAGA-3'	nt 324-345
	genotype C	5'-GGTCCTAGGAATCCTGATGTTG-3'	nt 165-186
	genotype ABC	5'-GGCTCMAGTTCMGAACAAGT-3'	nt 67-86
HBV Plasmid			
Full length	F-1818(<i>SacI</i>)	5'-CTATATTAAGCAGAGCTCCACCAGCACCATGCAACTTTTTC-3'	nt 1804-1826
	L-Reverse(<i>SapI</i>)	5'-CCGGAAAGCTTGAGCTCTTCAACACACCAATTTATGCCTAC-3'	nt 1783-1803
For sequencing			
	pLB forward	5'-CGACTCACTATAGGGAGAGCGGC-3'	pLB vector (HBV nt1804)
	P4	5'-CCTTGGACACATAAGGTGGG-3'	nt 2457-2476
	P5	5'-GTGGAGCCCTCAGGCTCAGG-3'	nt 3075-3094
	P6	5'-GCTGCTATGCCTCATCTTC-3'	nt 415-433
	P7	5'-CCAACTTACAAGGCCTTTC-3'	nt 1102-1120

2.2 Amplification of the full-length of HBV genome and sequencing analysis

To further verify the HBV genotype, the full-length of HBV genome was amplified using F-1818/L-reverse primer pairs using KOD DNA polymerase (TOYOBO, Japan, Cat#KMM101), which possesses proof-reading activity to minimize nucleotide mismatch (Table 1). The PCR products were recovered from the 1% agarose gel and ligated with the pLB-vector (Tiangen, Cat#VT205), and were subsequently transformed into DH5α *Escherichia coli*. Positive colonies detected by conventional colony PCR with genotype-specific primer pairs were further cultured in Luria Broth medium containing ampicillin (100 mg/mL). Plasmid DNA was isolated and subjected to the full-length HBV genome sequencing.

The obtained HBV sequences were genotyped using the NCBI web-based HBV genotyping tool (<https://www.ncbi.nlm.nih.gov/projects/genotyping/formpage.cgi>) (Rozanov et al. 2004).

3. Results

3.1 Phenomenon of the genotype B and C mixture

In the present study, we tested the accuracy of PCR-based HBV genotyping in 52 serum samples before commencing large scale HBV genotype screening. As a result, 55.8% (29 of 52) of the samples were determined to be HBV genotype B. Intriguingly, 44.2% (23 of 52) samples were determined as a mixture of genotype B and C based on the migration of PCR products in DNA electrophoresis. The representative pattern of DNA migration on electrophoresis is described in Figure 1A. Moreover, based on the DNA band density on agarose gel, the PCR production of genotypes B and C was variable in the serum samples; some samples produced more genotype B than C (Figure 1A, lane 3) or produced less genotype B than C (Figure 1A, lane 4).

3.2 Differentiation of mono- or co-infection of the HBV genotype

To verify whether the mixed genotype phenomenon was due to the co-infection of HBV genotype B and C, we randomly selected three genotype B samples and three mixed genotype samples for whole genome sequencing. The amplified whole HBV genome was further cloned into the pLB-vector. Next, ten colonies from each sample were selected and subjected to conventional colony-PCR using genotype-specific primers to verify the success of cloning and the HBV genotype. As a result, colonies from genotype B continued to be detected as genotype B (Figure 1B, lane 2) and colonies from the mixed genotype were detected as a mixture of genotype B and C (Figure 1B, lane 3). These findings suggest that the phenomenon of the genotype mixture was not the result of a co-infection with genotypes B and C.

3.3 Determination of the HBV genotype using the NCBI web-based HBV genotyping tool

To study the precise genotype of the cloned full-length HBV genome, one colony from each sample was further cultured in Luria Broth medium. The plasmid DNA was isolated and subjected to a sequencing analysis. The obtained HBV sequences were genotyped using the NCBI web-based HBV genotyping tool (<https://www.ncbi.nlm.nih.gov/projects/genotyping/formpage.cgi>). The primer pairs used for full-length sequencing are listed in Table 1. The obtained full-length HBV genome sequences were submitted to

GenBank (Accession Nos: MZ934422, MZ934424, MZ934425, MZ934423, MZ934426, and MZ934427). As a result, the mixed genotype samples (Accession Nos: MZ934422, MZ934424, and MZ934425) were all classified as genotype C, and the genotype B samples were all classified as genotype B.

3.4 Comparison of the genotype B-specific primer sequences

To compare the sequences of genotype B-specific primers between genotypes B and C, the reverse primer of genotype B was aligned with the six aforementioned sequences. Unlike the genotype B samples, which had almost identical sequences to the primer, genotype C had three nucleotide mismatches (Figure 2): one located at the 5' end of the primer sequence and two situated at the 3' end of the primer sequence.

Discussion

In the present study, we attempted to test the accuracy of a PCR-based HBV genotyping method in a small number of samples prior to initiating large scale HBV genotype screening. Intriguingly, the phenomenon of mixed genotypes was detected in 44.2% (23 of 52) of serum samples using genotype-specific primers (Naito et al. 2001). Since the genotype-specific primers were designed to detect a single genotype, we assumed that patients were co-infected with both genotypes B and C. To address this assumption, we randomly selected six samples and the full-length HBV genome was amplified, cloned, and transformed into *E.Coli*.

While the colony that should have contained one HBV genotype was subjected to genotype-specific PCR, it still showed the mixed genotype phenomenon, suggesting that it was not a genotype B and C co-infection. Based on the full-length HBV genome sequences and web-based genotyping, they all belonged to genotype C. Next, we assumed that the HBV genotype C DNA may contain highly homologous DNA sequences to the genotype B primer binding site. After comparing the genotype B specific primer sequences with the HBV genotype C DNA sequences, three nucleotide mismatches were found (Figure 2): one was located at the 5' end of the primer sequence and two were situated at the 3' end of the primer sequence. Although the three nucleotide mismatches, especially the mismatch at the 3' end of the primer, may reduce the primer binding efficiency, the genotype B-specific primer in our study continued to efficiently bind to HBV genotype C. This effect may result in the genotype B and C mixture phenomenon. In addition, although the six successfully amplified full-length genomes cannot represent all of the studied samples, the results suggest that detection of the HBV genotype via PCR-based methods may easily mislead the mono-infection or co-infection results of the HBV genotype.

Taken together, the present findings indicate that PCR-based HBV genotyping may not be accurate for the determination of the HBV genotype. Moreover, whole HBV genome sequencing remains the "gold standard" for HBV genotyping.

Declarations

Conflict of interest

All authors declare that they have no conflict of interest.

Research involving human participants and/or animals

This article did not contain any study with human participants or animals.

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Figures

Figure 1

Detection of HBV genotypes. A) HBV DNA templates in the serum. B) Conventional colony PCR. Abbreviations: M, DNA marker; B, genotype B; C, genotype C; B&C, a mixture of genotype B and C; bp, base pair.

Figure 2

Alignment of the genotype B-specific primer with a full-length genotype C sequence. Genotype C full-length genomes from the present study were aligned with the genotype B-specific primer (reverse primer). The mismatched nucleotides are indicated with a rectangular box.