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Improved multiplex-PCR to identify hepatitis B virus genotypes A–F and subgenotypes B1, B2, C1 and C2☆

Jinsong Chen [a], Jianhua Yin [a], Xiaojie Tan [a], Haiqin Zhang [a], Hongwei Zhang [a], Beichuan Chen [a], Wenjun Chang [a], Stephan Schaefer [b], Guangwen Cao [a,*]

[a] *Department of Epidemiology, Second Military Medical University, 800 Xiangyin Road, Shanghai 200433, PR China*

[b] *Abteilung für Virologie, Universität Rostock, Germany*

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Abstract

Background: There are eight genotypes (A–H) and numerous subgenotypes of hepatitis B virus (HBV). The genotype has been shown to affect the course of HBV infection.

Objectives: To develop an efficient genotyping and subgenotyping method for large-scale epidemiological surveys of HBV infection in countries with high prevalence of HBV B and C such as China.

Study design: We designed genotype and subgenotype-specific primer pairs, and adjusted PCR conditions for a multiplex-PCR using common *Taq* polymerase to identify HBV genotypes A–F in one reaction and for the main subgenotypes B1/B2 and C1/C2 in another reaction.

Results: We have developed a multiplex-PCR system, which specifically amplifies DNA of HBV genotypes and the corresponding main subgenotypes B and C from the sera of HBV patients. Our patients were infected with HBV of subgenotypes B2 ($n = 18$), C1 ($n = 2$) and C2 ($n = 48$). Eleven patients were doubly infected and three showed a triple infection with HBV A, B and C.

Conclusions: The low-cost multiplex-PCR for identification of HBV genotypes A–F and main subgenotypes of HBV B and C, is rapid, reliable and sufficient for large-scale epidemiological surveys and clinical studies.

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Keywords: Multiplex-PCR; Hepatitis B virus; Genotypes; Subgenotypes

1. Introduction

Hepatitis B virus (HBV) infection is a significant public health problem with more than 400 million people chronically infected around the world (McMahon, 2005). Seventy-five percent of these live in Asian countries (Zuckerman and Zuckerman, 2000).

HBV has been classified into eight genotypes A–H based on divergence over the entire HBV genomic sequence of greater than 8% (Kidd-Ljunggren et al., 2002; Kramvis et al., 2005; Norder et al., 2004; Okamoto et al., 1988; Schaefer, 2005). The clinical picture, the response to treatment, the long-term prognosis as well as seroconversion profile are influenced by the HBV genotype infecting the patient (Chu and Lok, 2002; Kramvis and Kew, 2005; Kao, 2002; Kao et al., 2000; Schaefer, 2005). China is a country seriously affected by the burden of chronic HBV infection. The prevalence of chronic HBV infection is 5–18% in mainland China (Kidd-Ljunggren et al., 2002; Kramvis and Kew, 2005; Norder et al., 2004; Okamoto et al., 1988).

The genotypes mostly prevalent in China are genotypes B and C (Zeng et al., 2005). Genotypes are further separated into subgenotypes if the divergence in nucleotide sequence

Abbreviations: HBV, hepatitis B virus; PCR, polymerase chain reaction; HCC, hepatocellular carcinoma; bp, base pairs

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* Corresponding author. Tel.: +86 21 25070420; fax: +86 21 25070420.
E-mail address: gcao@smmu.edu.cn (G. Cao).

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is between 4% and 8% (Kramvis et al., 2005; Norder et al., 2004). Up to now, HBV genotypes B and C are divided into at least five subgenotypes (Nagasaki et al., 2006; Sakamoto et al., 2006). The strains B1 and B2 (formerly named Bj and Ba, respectively) are predominant within genotype B. B1 is mainly found in Japan, whereas B2 is prevalent all over Asia (Sugauchi et al., 2002). HBV subgenotypes C1 (Cs) and C2 (Ce) are the predominant strains amongst the five subgenotypes of HBV genotype C (Chan et al., 2005; Huy et al., 2004; Sakamoto et al., 2006; Tanaka et al., 2005). Regarding the subgenotype designations for genotype C1 and C2 we follow the nomenclature suggested by Huy et al. (2004).

HBV subgenotypes C1 and C2, B1 and B2 show virological and epidemiological differences that may lead to an altered clinical picture in patients infected (Chan et al., 2005; Sugauchi et al., 2003).

Reliable and easy methods to differentiate HBV genotypes and subgenotypes are a prerequisite for molecular epidemiological tests and clinical studies. Several methods have been developed to identify HBV genotypes or subgenotypes (Bartholomeusz and Schaefer, 2004). Multiplex-PCR method appears suitable for large-scale analyses, however are costly due to expensive reagents (Kirschberg et al., 2004; Naito et al., 2001). In this study we have developed an effective multiplex-PCR system using common *Taq* polymerase, which is rapid and sensitive for identification of HBV genotypes A–F and suitable for the differentiation of subgenotypes B1, B2, C1 and C2.

2. Material and methods

2.1. Primer selection

For the primer selection, 362 complete sequences of HBV genotypes A–F were retrieved from GenBank, with at least 15 sequences for each genotype. A preference was given to HBV sequences submitted by laboratories from all over the world to account for geographic and demographic differences.

The PCR primers were designed on the basis of sequence alignment of each genotype by DNAStar and Primer Premier 5.0 softwares. In designing the primers, we searched for genotype-specific nucleotide sequences, which were conserved in each of the six HBV genotypes and the four HBV subgenotypes B1/B2 and C1/C2. Sequences within the same genotype or the same subgenotype were different by $\leq$2 nucleotides (usually $\leq$1 nucleotide) along the entire sequences of the primer, while the sequence within the different genotype or subgenotype had a difference of $\geq$2 nucleotides (usually $\geq$3 nucleotides). Primer sequences specific for each HBV genotype or subgenotype were further analyzed using the above software packages to obtain primer pairs with identical melting temperature and GC content. All of the primer pairs were validated and modified by using Primer Premier 5.0, Oligo 6.0 softwares.

2.2. Primer sequences and synthesis

The sequences of the HBV genotype-specific or subgenotype-specific primers are shown in Table 1. PAGE

Table 1
Specific primer pairs to amplify HBV genotypes A–F and subgenotypes of genotypes B and C

Genotype/subgenotype		DNA sequence (from 5′ to 3′)	Position	Size (bp) of product
A	A–S	AAACTACTGTTGTTAGACGACGRGACC	2334–2360	644
	A–AS	CTGGATTGTTTGARTTGGCTCCG	2955–2977	
B	B–S	CCAAACTCTTCAAGATCCCAGAGTCA	16–41	331
	B–AS	ACARGTTGGTGAGTGACTGGAGATTT	321–346	
C	C–S	CTCCCATCTCTCCACCTCTAAGAGACAGT	3164–3192	242
	C–AS	CAGGGGTCCTAGGAATCCTGATGTKG	165–190	
D	D–S	CAGACGCCAACAAGGTAGGAGCT	2972–2994	189
	D–AS	GAGTGTYTCTCAAAGGTGGAGACAGM	3135–3160	
E	E–S	ATACCCTATGGAAGGCGGGCATCT	2752–2775	130
	E–AS	CCCATTCGAGAGGGACCGTCCA	2860–2881	
F	F–S	TATCTGTGGGTATCCATTTGAATACYTC	815–842	487
	F–AS	CGAGCGAAACARGCTGCWAG	1282–1301	
B1	B1–S	CCTTCTGACTTCTTTCCGTCGG	1958–1979	237
	B1–AS	CCTGATCTTTAGGCCCATGTTAGTG	2170–2194	
B2	B2–S	GGGCTTTATTCTTCTACGGTACCTTG	2487–2512	278
	B2–AS	TTCCAAAGAGTGTGTAAATAATGTCKC	2738–2764	
C1	C1–S	CATTGTTCACCTCACCATACAGCACTA	2040–2066	554
	C1–AS	CATATTGTTGACATCTRTTGATAATATCCTCTTT	2560–2593	
C2	C2–S	ATACTCTGTGGAAGGCTGGCATTC	2752–2775	653
	C2–AS	GGGGTCCTAGGAATCCTGATGTTG	165–188	

S: sense, AS: anti-sense, R: G or A, K: T or G, M: A or C, W: A or T, Y: C or T.

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