Original Article

Hepatitis B Immunoprophylactic Failure and Characteristics of the Hepatitis B Virus Gene in Mother-Infant Pairs in Parts of China*

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Abstract

Objective To determine the hepatitis B immunoprophylactic failure rate in infants born to hepatitis B virus (HBV) infected mothers and to characterize HBV genes.

Methods HBV-serological testing was conducted for pregnant women and infants. The complete genomes of 30 HBV isolates were sequenced, and genetic characteristics were analyzed using MEGA 5 software.

Results The immunoprophylactic failure rate for infants who had completed the scheduled hepatitis B vaccination program was 5.76% (32/556). High sequence homology (99.8%-100%) was observed in 8 of the 10 mother-infant pairs. We identified 19 subgenotype C2 strains, 9 subgenotype B2 strains, and 2 subgenotype C1 strains. Three serotypes were detected: adr (19/30), adw (9/30), and ayw (2/30). The frequency of amino acid mutation of the 'a' determinant region was 16.67% (5/30), including that of Q129H, F134Y, S136Y, and G145E. We detected 67 amino acid mutations in the basal core promoter, precore, and core regions of the genome.

Conclusion The immunoprophylactic failure rate in infants born to HBV-infected mothers is low in the regions of China examined during this study. Moreover, HBV mutation in the 'a' determinant region could not account for immunoprophylactic failure for all infants.

Key words: Mother-to-infant transmission; Scheduled vaccination; Gene characteristics; 'a' determinant mutation; Immune escape

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INTRODUCTION

Hepatitis B virus (HBV) infection is a global health problem and is especially prevalent in developing regions. A national serological survey of hepatitis B was conducted in China in 2006, revealing that the prevalence of HBV surface antigen (HBsAg) was 2.1% among all children born during 1992-2005 and 1.0% among children born during 1999-2005[1]. There are three major modes of HBV transmission: (1) blood-borne, (2) sexual, and (3) mother-to-infant. In

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China, progress has been made in reducing blood-borne hepatitis B and C infection through use of disposable syringe needles, strict disinfection measures, and safe blood transfusions. Sexual transmission of HBV remains the major mode of transmission for some genotypes, especially in western areas of the world. However, in parts of Asia, including China, the major route of HBV transmission is vertical, i.e., mother to infant. Because of the higher tolerance levels of children's immune systems, HBV infection in children can more easily progress to chronic stages compared to infection in adults[2]. Immunoprophylaxis is the most effective method to prevent HBV infection, especially for newborns delivered by HBV-infected mothers. In China, scheduled hepatitis B vaccination in children was introduced in 1992 and further expanded in 2002. All newborns receive three doses of the hepatitis B vaccine at 24 h, 1 month, and 6 months after birth. Children born to HBV-infected mothers receive an additional dose of HBV immunoglobulin (HBIG) within 24 h of delivery. This study aims to determine the rate of immunoprophylactic failure in infants born to HBV-infected mothers in different parts of China.

HBV is a DNA virus belonging to the *Hepadnaviridae* family. There are at least nine genotypes (A-I) of HBV, and their classification is based on either an 8% divergence in the whole nucleotide sequence or a 4% divergence in the HBV surface gene[3-10]. Generally, divergence greater than or equal to 8% in the whole nucleotide sequence or 4% in the HBV surface gene will be considered new genotypes. Different genotypes vary in geographical distribution[11], mode of transmission (vertical or horizontal), and response to antiviral drugs[12-14]. In southern China, the dominant HBV genotype is B, whereas genotype C predominates in northern areas[15]. The main route of transmission for both of these genotypes (B and C) is vertical. There are four open reading frames in the HBV genome, which encode several functional or structural proteins. Although HBV is a DNA virus, it replicates via an RNA intermediate[16]. The poor proofreading ability of the reverse transcriptase results in significant genetic diversity across various HBV genotypes. Nucleotide or amino acid substitutions in the HBV surface gene, especially in the 'a' determinant region, often result in immune escape, immunoprophylactic failure, and missed diagnosis[17-18]. Several recognized T cell and B cell epitopes are located in the core gene region. Nucleotide or amino acid substitutions in the basal core promoter (BCP), precore, and core regions of the genome can usually predict disease severity[19-21]. The aims of this study were to characterize HBV homology, genotypes, serotypes, and the nucleotide or amino acid substitutions in the surface gene, BCP, precore, and core regions in mothers and infants and to describe any trends in these characteristics associated with immunoprophylactic failure.

METHODS

Study Sites and Population

In total, seven provincial or county-level hospitals across three Chinese provinces (Jilin, Sichuan, and Gansu) were chosen for this study. The field study occurred from June 2013 to February 2015. HBsAg-positive pregnant women and their infants were recruited from the study site locations. The study protocol was approved by the Chinese Center for Disease Control and Prevention, and informed consent was obtained from all of the adult participants.

HBV Serological and DNA Load Tests for Mothers

HBV serological testing and blood collection were performed on mothers with their consent after admission to the hospital for child delivery. Immunoassay tests detected HBsAg, antibodies to the HBV surface antigen (anti-HBs), HBV envelope antigen (HBeAg), antibodies to the HBV envelope antigen (anti-HBe), and antibodies to the HBV core antigen (anti-HBc). The Micro-Particle Enzyme Immunoassay Kit for HBV serological testing was produced by Abbott Laboratories. Quantitative polymerase chain reaction was performed to measure HBV DNA load for HBsAg-positive women using a kit named careHBV PCR Assay V3 produced by QIAGEN.

Scheduled Hepatitis B Immunoprophylaxis Programs for Infants

A full course of vaccinations was carried out for all infants in our study, including three doses of recombinant yeast-derived or Chinese hamster ovary cell-derived hepatitis B vaccines administered within 24 h and again at 1 month and 6 months after birth. Infants with HBsAg-positive mothers also received a single dose of HBIG within 24 h.

HBV Serological Tests for Infants

Blood samples were collected from infants

between 4 and 6 months after the completion of their HBV prophylaxis. Five HBV serological markers were tested.

HBV Whole Genome Sequencing and Analysis of HBV Genetic Characteristics in Mother-Infant Pairs

Infants who showed a serum anti-HBs titer lower than 10 mIU/mL but were serum positive for at least one of the four HBV markers (HBsAg, HBeAg, anti-HBe, and anti-HBc) were defined as experiencing immunoprophylactic failure. Viral DNA was extracted from samples of these infants and their mothers (Viral Blood DNA Mini Kit; QIAGEN) according to the manufacturer's instructions. The HBV genome was amplified using PCR. Primers and the amplification profile used here were identical to that employed by Günther et al.[22]. PCR products were purified (DNA Fragment Purification Kit Ver. 2.0; TaKaRa Biotechnology) and directly sequenced using an Applied Biosystems 3730 sequencer. The HBV sequences of mothers and infants were aligned and compared with the HBV reference sequences for all nine genotypes, downloaded from GenBank (Accession numbers provided in Figure 1). A phylogenetic tree was constructed using Mega Software version 5.0, applying the Kimura two-parameter matrix and the maximum likelihood method. To test the phylogeny predictions, bootstrap replications were carried out 500 times. Genotypes, serotypes, and nucleotide or amino acid substitutions in the surface, BCP, precore, and core gene regions were determined.

RESULTS

Immunoprophylactic Failure Rate

During a period of 20 months (June 2013 to February 2015), 595 pregnant women who were suspected of being infected with HBV were admitted to various hospitals within the study area and consented to HBV serological testing. Those who met the inclusion criteria were admitted to the study, leaving a final number of 556 HBsAg-positive pregnant women. There were 289 women participants in Sichuan, 117 in Jilin, and 150 in Gansu province. The serological profiles for the women in our study were as follows: 150 were HBsAg(+), HBeAg(+), and anti-HBc(+); 335 were HBsAg(+), anti-HBe(+), and anti-HBc(+); and 71 were HBsAg(+) and anti-HBc(+). For HBeAg(+) pregnant women, the HBV DNA load before child delivery was 8.90×10^{3}-1.60×10^{9} IU/mL, with an average value of 6.10×10^{7} IU/mL. For anti-HBe positive pregnant women, the average viral DNA load was 3.12×10^{6} IU/mL, with a range of 0 to 2.8×10^{8} IU/mL. For HBsAg and anti-HBc-positive women, the HBV DNA loads ranged from 0 to 2×10^{6} IU/mL, with an average of 6.63×10^{4} IU/mL (Table 1).

A total of 558 infants were recruited to our study, including two pairs of twins. The results of their HBV serological tests indicated that the 32 infants, including one pair of twins, were experiencing immunoprophylactic failure. The total immunoprophylactic failure rate for this study was 5.76% (32/556), with a provincial failure rate of 2.77% in Sichuan, 15.38% in Jilin, and 4.00% in Gansu. In infants whose mothers were HBeAg(+), the immunoprophylactic failure rate was 8.67% (13/150), while it was 5.67% (19/335) in the anti-HBe(+) group. There were no HBsAg(+) infants born to mothers with serological profile of 'HBsAg(+), anti-HBc(+)'. The immunoprophylactic failure rate for infants whose mother was HBeAg(+) was significantly higher than that of other groups (Chi-square test: P=0.0354).

Twenty-four infants showed negative results for five of the HBV markers, with a total unresponsiveness rate of 4.32% (24/556). In infants whose mothers were HBeAg(+), the unresponsiveness rate was 2.00% (3/150), while it was 5.97% (20/335) in the anti-HBe(+) group and 1.41% (1/71) in infants born to HBsAg(+) and anti-HBc(+) mothers. Moreover, there was no statistical difference among the three groups (Chi-square test: P=0.0602).

Clinical, virological, and biochemical characteristics of the mothers whose infants were experiencing immunoprophylactic failure are shown in Table 2. Thirty-one mothers aged from 20 to 34 years (average age: 25.2±2.9 years), with 2-18 years of HBV infection history. All 31 mothers experienced no clinical symptoms related to liver disease and did not consent any anti-HBV treatment during pregnancy. The HBV DNA level and ALT (alanine aminotransferase) level of mothers are provided in Table 2. Four serological profiles were detected in their infants: (1) anti-HBe(+) and anti-HBc(+) (16/32); (2) HBsAg(+), HBeAg(+), and anti-HBc(+) (11/32); (3) HBsAg(+) and anti-HBc(+) (4/32); and (4) HBsAg(+) (1/32).

Phylogenetic Tree and Homology Analysis

PCR and sequencing were successful for 30 subjects

Table 1. The Immunoprophylactic Failure Rates and Unresponsiveness Rates of Different Serological Profiles

Province	Mother's Serological Profile	No. of Mothers	Mean of HBV DNA (IU/mL)	No. of Immunoprophylactic Failure Infants	Failure Rate (%)	*P* Value	No. of Unresponsiveness Infants	Rate of Unresponsiveness (%)	*P* Value
Sichuan	HBsAg(+), HBeAg(+), anti-HBc(+)	65	5.86×10^{7}	5	7.69		0	0.00	
	HBsAg(+), anti-HBe(+), anti-HBc(+)	181	3.18×10^{6}	3	1.66		11	6.08	
	HBsAg(+), anti-HBc(+)	43	6.77×10^{4}	0	0.00		0	0.00	
	Total	289	1.52×10^{7}	8	2.77	0.0365*	11	3.81	0.0339*
Jilin	HBsAg(+), HBeAg(+), anti-HBc(+)	42	6.12×10^{7}	5	11.90		3	7.14	
	HBsAg(+), anti-HBe(+), anti-HBc(+)	59	3.71×10^{6}	13	22.03		5	8.47	
	HBsAg(+), anti-HBc(+)	16	8.34×10^{4}	0	0.00		0	0.00	
	Total	117	2.39×10^{7}	18	15.38	0.0705**	8	6.84	0.6909*
Gansu	HBsAg(+), HBeAg(+), anti-HBc(+)	43	6.44×10^{7}	3	6.98		0	0.00	
	HBsAg(+), anti-HBe(+), anti-HBc(+)	95	2.64×10^{6}	3	3.16		4	4.21	
	HBsAg(+), anti-HBc(+)	12	3.85×10^{4}	0	0.00		1	8.33	
	Total	150	2.04×10^{7}	6	4.00	0.6247*	5	3.33	0.1637*
Total	HBsAg(+), HBeAg(+), anti-HBc(+)	150	6.10×10^{7}	13	8.67		3	2.00	
	HBsAg(+), anti-HBe(+), anti-HBc(+)	335	3.12×10^{6}	19	5.67		20	5.97	
	HBsAg(+), anti-HBc(+)	71	6.63×10^{4}	0	0.00		1	1.41	
	Total	556	1.83×10^{7}	32	5.76	0.0354**	24	4.32	0.0602**

Note. *Fisher's exact test was used. **Chi-square test was used.

Table 2. Clinical, Virological, and Biochemical Characteristics of Mothers Whose Infants Displayed Immunoprophylactic Failure

Pair	Member	Age (years)	Self-reported HBV Infection History (years)	Clinical Symptoms	Serological Profile	HBV DNA (IU/mL)	ALT (IU/L)	Infant's Serological Profile
1*	Mother	20	2	No	HBsAg(+), HBeAg(+), anti-HBc(+)	4.31×10^{7}	53	For one infant: HBsAg(+), HBeAg(+), anti-HBc(+); for the other: all negative.
2*	Mother	22	2	No	HBsAg(+), HBeAg(+), anti-HBc(+)	1.73×10^{6}	46	HBsAg(+), HBeAg(+), anti-HBc(+)
3*	Mother	26	2	No	HBsAg(+), HBeAg(+), anti-HBc(+)	7.99×10^{7}	29	HBsAg(+)
4*	Mother	25	3	No	HBsAg(+), HBeAg(+), anti-HBc(+)	7.32×10^{7}	73	HBsAg(+), HBeAg(+), anti-HBc(+)
5*	Mother	25	3	No	HBsAg(+), HBeAg(+), anti-HBc(+)	3.51×10^{7}	60	HBsAg(+), HBeAg(+), anti-HBc(+)
6*	Mother	22	3	No	HBsAg(+), anti-HBe(+), anti-HBc(+)	8.56×10^{2}	17	HBsAg(+), HBeAg(+), anti-HBc(+)
7*	Mother	28	3	No	HBsAg(+), HBeAg(+), anti-HBc(+)	1.56×10^{6}	45	HBsAg(+), HBeAg(+), anti-HBc(+)
8*	Mother	22	3	No	HBsAg(+), anti-HBe(+), anti-HBc(+)	2.70×10^{2}	86	HBsAg(+), HBeAg(+), anti-HBc(+)
9*	Mother	26	3	No	HBsAg(+), HBeAg(+), anti-HBc(+)	1.97×10^{6}	20	HBsAg(+), HBeAg(+), anti-HBc(+)
10*	Mother	28	18	No	HBsAg(+), anti-HBe(+), anti-HBc(+)	3.00×10^{3}	9	The twin infants had the same profile: HBsAg(+), HBeAg(+), anti-HBc(+)
11	Mother	23	3	No	HBsAg(+), HBeAg(+), anti-HBc(+)	2.86×10^{6}	45	anti-HBe(+), anti-HBc(+)
12	Mother	25	2	No	HBsAg(+), HBeAg(+), anti-HBc(+)	5.32×10^{6}	48	anti-HBe(+), anti-HBc(+)
13	Mother	27	5	No	HBsAg(+), anti-HBe(+), anti-HBc(+)	1.09×10^{3}	13	anti-HBe(+), anti-HBc(+)
14	Mother	28	5	No	HBsAg(+), anti-HBe(+), anti-HBc(+)	8.54×10^{1}	27	anti-HBe(+), anti-HBc(+)
15	Mother	23	3	No	HBsAg(+), anti-HBe(+), anti-HBc(+)	9.68×10^{2}	15	anti-HBe(+), anti-HBc(+)
16	Mother	21	4	No	HBsAg(+), anti-HBe(+), anti-HBc(+)	2.32×10^{3}	35	anti-HBe(+), anti-HBc(+)
17	Mother	24	2	No	HBsAg(+), anti-HBe(+), anti-HBc(+)	1.91×10^{2}	19	anti-HBe(+), anti-HBc(+)
18	Mother	28	4	No	HBsAg(+), anti-HBe(+), anti-HBc(+)	3.14×10^{2}	14	anti-HBe(+), anti-HBc(+)
19	Mother	26	7	No	HBsAg(+), anti-HBe(+), anti-HBc(+)	2.05×10^{1}	8	anti-HBe(+), anti-HBc(+)
20	Mother	25	6	No	HBsAg(+), anti-HBe(+), anti-HBc(+)	1.48×10^{1}	12	anti-HBe(+), anti-HBc(+)
21	Mother	29	10	No	HBsAg(+), anti-HBe(+), anti-HBc(+)	2.35×10^{6}	55	anti-HBe(+), anti-HBc(+)
22	Mother	25	5	No	HBsAg(+), anti-HBe(+), anti-HBc(+)	2.01×10^{3}	21	anti-HBe(+), anti-HBc(+)
23	Mother	22	3	No	HBsAg(+), anti-HBe(+), anti-HBc(+)	2.78×10^{2}	15	anti-HBe(+), anti-HBc(+)
24	Mother	23	2	No	HBsAg(+), anti-HBe(+), anti-HBc(+)	1.56×10^{2}	9	anti-HBe(+), anti-HBc(+)
25	Mother	26	4	No	HBsAg(+), anti-HBe(+), anti-HBc(+)	1.98×10^{2}	11	HBsAg(+), anti-HBc(+)
26	Mother	27	12	No	HBsAg(+), anti-HBe(+), anti-HBc(+)	4.83×10^{1}	32	HBsAg(+), anti-HBc(+)
27	Mother	29	16	No	HBsAg(+), HBeAg(+), anti-HBc(+)	3.82×10^{2}	22	HBsAg(+), HBeAg(+), anti-HBc(+)
28	Mother	22	4	No	HBsAg(+), HBeAg(+), anti-HBc(+)	5.69×10^{7}	78	anti-HBe(+), anti-HBc(+)
29	Mother	25	2	No	HBsAg(+), anti-HBe(+), anti-HBc(+)	7.22×10^{2}	9	HBsAg(+), anti-HBc(+)
30	Mother	34	15	No	HBsAg(+), HBeAg(+), anti-HBc(+)	1.33×10^{1}	50	HBsAg(+), anti-HBc(+)
31	Mother	26	3	No	HBsAg(+), HBeAg(+), anti-HBc(+)	7.40×10^{2}	12	anti-HBe(+), anti-HBc(+)

Note. *Complete HBV sequences from both mothers and infants were successfully sequenced.

(including one pair of twins), consisting of 10 mother-infant pairs and another 9 mothers. Sequences from each pair clustered in a distinct branch of the phylogenetic tree, and the bootstrap values ranged from 70% to 100%. Three subgenotypes were detected: 19 strains belonged to subgenotype C2, 9 strains to subgenotype B2, and 2 strains to subgenotype C1. The subgenotype of sequences obtained from infants was the same as that of their mothers (Figure 1).

With the exception of pairs 6 and 8, homologies of 99.8%-100% were observed between HBV strains isolated from mothers and their infants, with nucleotide changes at no more than five positions. Of the 30 HBV strains isolated, 28 were 3,215 bps long, while the maternal strain from pair 6 was 2,984 bps long and the maternal strain from pair 18 was 3,173 bps long. Poor homology was observed in the sequences from mothers and infants for pair 6 and 8, with only 92.4% and 99.2% homology, respectively. When compared to the sequences from pair 6, 12 nucleotide substitutions and a 231-nucleotide deletion were detected in the maternal strain. There were 26 nucleotide differences between the maternal and infant strains of pair 8.

Surface Gene Analysis

Three serotypes were detected, including adr (19/30), adw (9/30), and ayw (2/30). Strains from subgenotype B2 belonged to serotype adw, C2 strains were adr or ayw, and C1 strains were adr. Twenty (66.67%, 20/30) strains showed amino acid mutations in the surface gene region (nucleotides 155 to 835), with 52 amino acid mutations. Eight amino acid mutations located in the 'a' determinant region were detected in 5 (16.67%, 5/30) strains, including Q129H, F134Y, S136Y, and G145E (Table 3). In the HBeAg(+) group, 10 (55.56%, 10/18) strains showed amino acid mutations in the surface gene; whereas in the anti-HBe(+) group, amino acid mutations in the surface gene were detected in 10 (90.90%, 10/11) strains. However, the frequencies of amino acid mutations in the surface gene of the two groups were not statistically different (Fisher's Exact Test: P=0.0959).

HBV strains from pairs 1 and 2 had wild type surface genes. The mother of pair 1 was positive for HBeAg and the DNA load was 4.3×10^7 IU/mL. Although the twin girls from pair 1 received the same scheduled hepatitis B vaccination and HBIG, one of them experienced immunoprophylactic failure, while the other showed unresponsiveness to HBV vaccination.

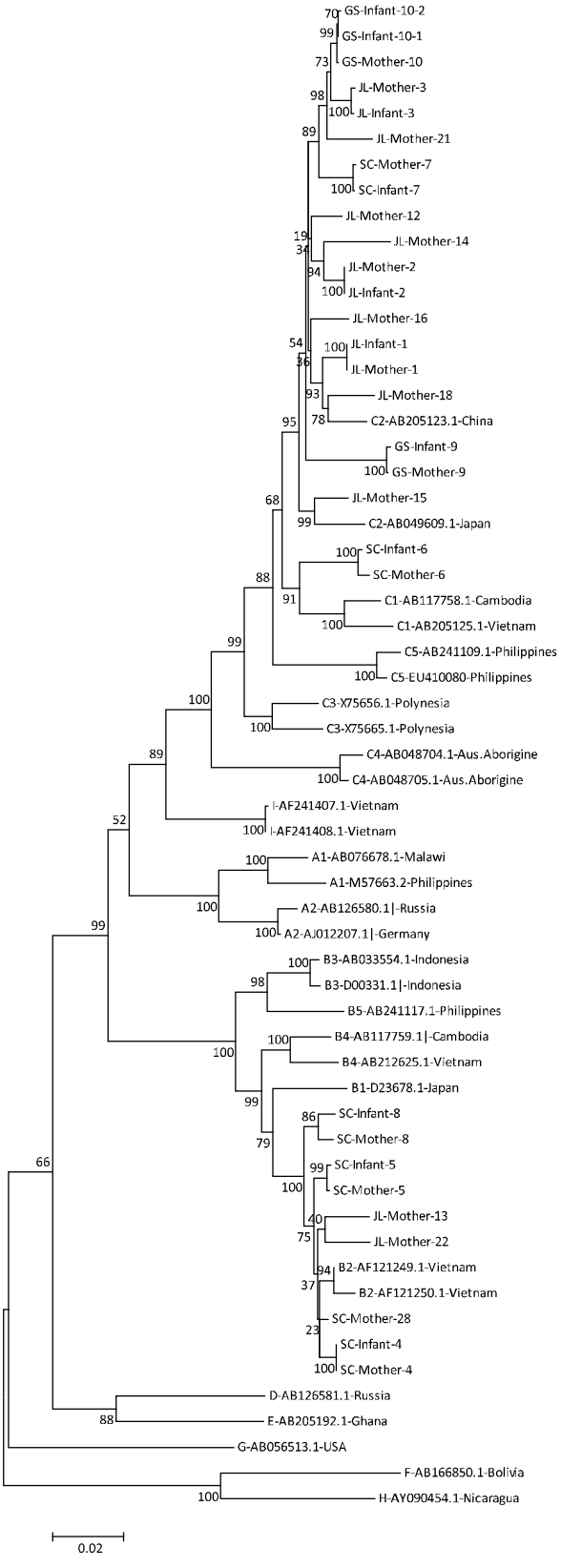

Figure 1. Phylogenetic tree of complete HBV sequences from mothers and infants.

Table 3. HBV Gene Characteristics of Mothers and Infants with Immunoprophylactic Failure

Pair	Member	Sub-genotype	Serotype	Amino Acid Substitutions in Surface Gene Region	Nucleotide (amino acid) Mutations in BCP, Precore and Core Gene Region
1	Mother	C2	adr	-	-
	Infant	C2	adr	-	-
2	Mother	C2	adr	-	-
	Infant	C2	adr	-	-
3	Mother	C2	adr	T123A	-
	Infant	C2	adr	-	-
4	Mother	B2	adw	L/M213I	-
	Infant	B2	adw	L/M213I	-
5	Mother	B2	adw	-	-
	Infant	B2	adw	G10R	-
6	Mother	C1	adr	S53L, I68T, V194A	231bps deletion, A1762T/G1764A, C1913A(P34T), T2012C(Y67H)
	Infant	C1	adr	S53L, I68T, V194A	-
7	Mother	C2	adr	-	-
	Infant	C2	adr	-	-
8	Mother	B2	adw	V14A, G44E, S61L	G1896A(W28STOP), C1914A/G1915C(P34H), T2003A(S64T), A2159G(S116G)
	Infant	B2	adw	G44E, S61L	G1915C(P34H), A2159G(S116G)
9	Mother	C2	ayw	P46T, I68T, F134Y, A159G, V168A, N207S	-
	Infant	C2	ayw	P46T, I68T, F134Y, A159G, V168A, N207S	-
10	Mother	C2	adr	V194A	-
	Infant-1	C2	adr	Q129H, F134Y, V194A	-
	Infant-2	C2	adr	Q129H, F134Y, V194A	-
12	Mother	C2	adr	-	-
13	Mother	B2	adw	G44E, S204R	A1762T/G1764A, G1896A(W28STOP), G1937A/T1938C(V42T)
14	Mother	C2	adr	T47A	T1934A(S41T), A1979G/T1981A(I56V), T2010A(L66Q), G2020T(E69D), A2100C(N96T), A2120G/G2121T/T2122A(S103V), T2126A(L105M), A2149C(E112D), G2171A/T2172C(V120T), A2189T(I126F), C2246A(L145I), C2337A(T175N)
15	Mother	C2	adr	V184A, P203R	A1762T/G1764A, A2189C(I126F), C2198A(L129I), C2288A(P159I), C2304A(P164Q), G2352A(R180Q), G2345A(V178I)
16	Mother	C2	adr	S53L	C2063A(L84I), A2159G(S116G), G2352A(R180Q), T2363C(S184P)
18	Mother	C2	adr	T47K, S136Y, G145E, P188H, L216STOP, I218T	A1762T/G1764A, G1896A(W28STOP), A1979G/T1981A(I56V), G2088T(G92V), A2120G(S103G), C2288A/C2290T(P159T)
21	Mother	C2	adr	G44E, S210R	A1762T/G1764A, G1896A(W28STOP), A1979G/T1981A(I56V), T1961G(S50A), T2003A/C2004A(S64N), C2048T(P79S), C2063A(L84I), G2092T(E93D), T2101A(N96T), G2105A(A98T), G2132C(D107H), G2160A(S116N), A2189T(I126F), A2239C(E142D), C2288A/C2289T(P159I)
22	Mother	B2	adw	L21S, S210R, M213I, F220L	A1762T/G1764A, G1896A(W28STOP), A1997T(T62S), A2013T(Y67F), T2042C/G2043T(C77L), A2131C(E106D), A2239T(E142D), C2304A(P164Q), A2339G(T176A), G2357T(G182C)
28	Mother	B2	adw	-	-

Sequences from pair 3 showed a T123A amino acid substitution in the maternal strain, which was not present in the viral strain from her infant. In pair 4, the maternal strain had a point mutation at nucleotide 639, changing the amino acid L/M213I, and an identical substitution was found in the viral strain of her infant. In pair 5, there was an amino acid substitution (G10R) in the infant strain only. In pair 6, identical substitutions were found in both the mother and the infant, including S53L, I68T, and V194A. In pair 7, both of the surface genes were wild type, and no substitutions were found in any of the strains. In pair 8, substitutions of G44E and S61L were found in both maternal and infant sequences, with an additional substitution (V14A) found in the maternal strain. In pair 9, the surface genes were highly variable, with six substitutions (P46T, I68T, F134Y, A159G, V168A, and N207S) identified. Both the mother and the infant in pair 9 were HBeAg(+).

Pair 10 comprised a mother and her twin boys, with all of the HBV strains identified belonging to subgenotype C2. The mother was 28 years old, positive for anti-HBe and had an HBV DNA load of 3.00×10^3 IU/mL before child delivery. Both infants were infected with HBV: one was HBeAg(+), and the other was anti-HBe(+). There was one substitution, V194A, in the surface gene of the mother and her twins. However, nucleotide changes, A541C and T555A, created two additional amino acid substitutions, Q129H and F134Y, which were observed in the twins.

For nine of the pairs, only the maternal HBV strains were successfully sequenced. Amino acid mutations of S136Y and G145E, which were located in the 'a' determinant, were detected in maternal strain of pair 18. In pair 18, the mother's serological profile was HBsAg(+), anti-HBe(+), and anti-HBc(+) and subgenotype C2. The infant from pair 18 had a serological profile of anti-HBe(+) and anti-HBc(+); however, the identity of the HBV strain from the infant was not successfully determined.

Mutations in BCP, Precore, and Core Regions of the Genome

Double mutations of A1762T/G1764A were detected in the BCP region of six strains, as shown in Table 3. The total mutation rate of A1762T/G1764A was 20.00% (6/30), while it was 0.00% (0/18) in the HBeAg(+) group and 54.54% (6/11) in the anti-HBe(+) group, respectively. The frequency of A1762T/G1764A mutations in anti-HBe(+) group was statistically higher than the HBeAg(+) group (Fisher's Exact Test: $P<0.05$).

Missense mutations in regions of the precore and core genes were found in 10 (33.33%) of the 30 strains. For the HBeAg-positive group, the rate of amino acid mutation was 5.56% (1/18), while it was 81.82% (9/11) in the anti-HBe-positive group. The missense mutation rate in the anti-HBe-positive group was higher than that in the HBeAg-positive group based on our statistical analysis (Fisher's Exact Test: $P<0.05$).

Missense mutations in the BCP, precore, and core genomic regions were detected in pair 6 and 8. In pair 6, the mother was 22 years old and her HBV DNA load was 855 IU/mL before delivery. She was anti-HBe(+) with normal ALT (17 IU/L) and remained asymptomatic for liver disease during pregnancy. Despite scheduled hepatitis B vaccinations and application of the HBIG program, her infant was also infected with HBV. The HBV sequence from the mother was 2984 bps long, with a 231 bps deletion between nucleotide 2068 to 2298. Double mutations at A1762T and G1764A were detected in the BCP region of the maternal strain but not in the infant strain. Point mutations of C1913A and T2012C in the core region created amino acid substitutions of P34T and Y67H in the maternal strain, but these were not detected in the infant.

In pair 8, four different amino acid mutations were detected. The mother had suffered from liver disease for two years, with positive anti-HBe, and maintained a high ALT (86 IU/L) level and a low HBV DNA load (270 IU/mL). In the maternal strain, we identified a G1896A mutation, which resulted in a stop codon at amino acid position 28 in the precore region. Sequencing revealed nucleotide mutations, C1914A and G1915C, which resulted in an amino acid substitution of P34H in the core region. We also identified nucleotide mutations, T2003A and A2159G, which resulted in two additional amino acid substitutions, S64T and S116G, in the same genomic region. However, only the P34H and S116G mutations were detected in the infant.

DISCUSSION

This study reports that the hepatitis B immunoprophylactic failure rate in infants born to HBV-infected mothers is low in the examined regions of China. The immunoprophylactic failure rate was 5.76% in our study, which is similar to the results from other studies conducted in China (3.40%-4.82%)[23-24]. Timely vaccination and

immunization with HBIG is very effective in interrupting HBV vertical transmission, with an efficacy of up to 94.24% (524/556) at our study sites. As the main mode of HBV transmission in China is vertical, medical treatment of HBV-infected mothers is a crucial component of controlling HBV infection. Most of the HBV-infected mothers in this study were born in the late 1980s or early 1990s-before widespread application of scheduled hepatitis B immunoprophylactic programs in China. The use of effective immunoprophylaxis program sharply decreased the rate of HBV infection for the next generation and HBV transmission should continue to decrease in the following generation. As a result of their immature immune systems, hepatitis B infection in children can more frequently progress to a chronic disease state[2]. Therefore, it is imperative to have a highly effective system in place for preventing vertical transmission, which should ultimately reduce the heavy economic burden of HBV-related liver disease in China.

HBeAg status and HBV DNA load are good predictors of risk for immunoprophylactic failure in pregnant women, which is consistent with the findings of others[2,23]. In our study, 87.23% (485/556) of the mothers were positive for HBeAg or anti-HBe, indicative of chronic HBV infection. Our results show that children of HBeAg-positive mothers have the highest immunoprophylactic failure rate-up to 8.67%. In contrast, this failure rate was reduced in the infants of anti-HBe-positive mothers, and there was a significantly reduced risk of immunoprophylactic failure in the infants of HBsAg(+) and anti-HBc(+) mothers. HBeAg and anti-HBe usually correlate with the HBV DNA level[25], which is consistent with our observations. The average HBV DNA load was very high in HBeAg(+) mothers but was significantly lower in anti-HBe(+) mothers. Thus, determination of HBeAg/anti-HBe status and HBV DNA level is critical for addressing HBV transmission in pregnant women. In China, many pregnant women do not know that they are infected with HBV until admission to a hospital for delivery of their child. We recommend that hepatitis B screening should be implemented in young women prior to pregnancy. For women chronically infected with HBV, HBeAg sero-conversion or treatment with antivirals that decrease HBV DNA load prior to pregnancy may decrease the risk of vertical transmission.

In our study, the total rate of unresponsiveness to HBV vaccination in infants born to HBV-infected mothers is low (up to 4.32%). In some studies in China, the rate of unresponsiveness to HBV vaccination for infants was 0.74% or 1.72%[26-27], which was lower than the rate obtained in our study for infants born to HBV-infected mothers. Unresponsiveness to HBV vaccination is correlated with several factors, such as vaccination conditions, virus subtype, and genetic background of the host. Infants whose anti-HBs titer was lower than 10 mIU/mL in our study were advised to undergo HBV vaccination again.

HBV vertical transmission accounted for a large proportion of infants who experienced immunoprophylactic failure. For 80% (8/10) of the mother-infant pairs, HBV sequences from infants had very high homology with their mothers, with the same subgenotype and serotype. This provides convincing molecular evidence that the HBV strains present in the infants originated from their mothers. HBV vertical transmission may occur during pregnancy, intrapartum, or postpartum. Because the HBIG and antibodies induced by the hepatitis B vaccine cannot neutralize the preexisting HBV in the body of these infants, intrauterine infection is considered to be an important risk factor in immunoprophylactic failure[28-30]. In our study, limited surveillance data meant that the time of exposure was not clear, revealing the need for further studies.

Horizontal transmission could be responsible for some of the incidents of HBV infection detected in infants in our study. Comparison of sequences from pairs 6 and 8 demonstrated large nucleotide differences between maternal and infant strains. Although they were of the same genotypes and serotypes, poor homology between HBV strains isolated from the mother and her infant does not support vertical transmission. However, the HBV quasispecies theory suggests that there is a population of HBV strains that co-exist in the body[31], with the dominant strain more easily detected by first-generation sequencing technology. If correct, this suggests that the strains observed in these infants might still have been the result of vertical transmission, with infection resulting from a less dominant strain of HBV. However, further study is needed to test this hypothesis and to explore HBV transmission using next-generation sequencing technologies, which are more sensitive and may be able to detect the presence of HBV quasispecies in the mother. At this point, however, the horizontal transmission route cannot be ruled out. Moreover, although the rate of horizontal transmission during

childbirth in China is unknown, strict management of hospital disinfection and sterilization should be carried out to minimize the risk of hospital-associated infections.

HBV is a DNA virus, but it replicates via an RNA intermediate[16]. The poor proofreading ability of the reverse transcriptase results in the majority of the genetic diversity observed in HBV. Nucleotide or amino acid substitutions in the HBV surface gene, especially in the 'a' determinant region, lead to immune escape, immunoprophylactic failure, and missed diagnosis[17-18]. We analyzed 30 HBV sequences from mothers and infants, and 20 (66.67%) had amino acid substitutions in the surface gene region. Fifty-two amino acid substitutions were found in the surface gene. A mutation at T123A was identified in our study; notably, a previous study reported that this mutation can lead to a loss of detection by immunoassays[32]. We also detected amino acid substitutions in the 'a' determinant region of the HBV surface antigen in 5 (16.67%) of the strains, including the previously characterized mutations Q129H, F134Y, S136Y, and G145E. Among the 11 strains sequenced from infants, 3 strains showed amino acid substitutions of Q129H and F134Y in the 'a' determinant region, which were reported to be 'immune escape' mutations[33-36]. Thus, we suggest that Q129H and F134Y detected in the twins from pair 10 may be correlated with HBV antibody escape, resulting in immunoprophylactic failure. However, based on the low rate of 'a' determinant mutations, we conclude that these mutations could not account for all of the immunoprophylactic failures observed in this study. Our results show that the mutation rate in the surface gene were not significantly different between the groups HBeAg(+) and anti-HBe(+).

In our study, the sequences of wild type and variant HBV remained stable during vertical transmission from mothers to infants, with only a handful of very slight changes observed. This also includes the strains isolated from the twins in pair 10 in which both obtained mutations of Q129H and F134Y that were not observed in their mother. High homology and similar mutations between mothers and infants strongly support the mother-to-infant transmission route. We infer that HBV may change slightly after entering a new host, thereby allowing it to better adapt to the new environment.

Three (14.3%) of the 21 HBV strains had amino acid substitutions in BCP, precore, and core regions, which may also help predict disease severity[19-21]. BCP is located between positions 1744 and 1804 and controls the production of precore RNA and core RNA. Examining the maternal strain of pair 6, we detected this double mutation and the patient was also HBeAg negative. The precore gene is located between nucleotides 1814 and 1900 and encodes 29 amino acids that constitute the precore protein. A G1896A mutation and a negative HBeAg test were observed in the mother in pair 8. The HBV core gene is located between nucleotides 1901 and 2452, encoding a structural protein called HBcAg. The HBcAg protein consists of 183 amino acids, and several known B- and T-cell epitopes are located in the region. Observation of the double mutation A1762T/G1764A, a 231 bps deletion, and the amino acid substitutions P34T and Y67H in the core gene of a single maternal strain demonstrates the high variability of circulating HBV. Moreover, this mother also had normal ALT levels and showed low viremia during pregnancy; thus, the medical history of this patient should be further examined.

Notably, the mutation rate of A1762T/G1764A in the anti-HBe(+) group was significantly higher than the HBeAg(+) group. Previous studies of BCP mutations occurring in Japanese patients revealed a correlation between the e antigen-negative phenotype and the frequently observed double mutation, A1762T/G1764A[37-38]. Similarly, the mutation rate in precore and core genes was higher in the anti-HBe(+) than that in the HBeAg(+) group.

In conclusion, we determined the rate of immunoprophylactic failure in infants born to HBV-infected mothers in several regions of China. Our results strongly support the vertical transmission mode of HBV infection, and we examined nucleotide and amino acid substitutions in the surface, BCP, precore, and core genomic regions. The rate of immunoprophylactic failure in infants born to HBV-infected mothers is low in the parts of China that we examined during our study. Timely vaccination and immunization with HBIG is very effective in interrupting HBV vertical transmission. Infants born to mothers who are positive for HBeAg and have high HBV DNA loads have an increased risk for immunoprophylactic failure. We recommend that women of childbearing age undergo hepatitis B screening prior to pregnancy. High levels of homology and similar nucleotide and amino acid substitutions between HBV sequences from infants and mothers strongly support the vertical transmission route. The low rate of 'a' determinant mutations suggests that these mutations cannot

account for all immunoprophylactic failures in infants. In our study, the time of exposure for infected infants remains unknown, which should be further addressed in future studies.

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