

Molecular Characteristics of HBV Among Blood Donors in Southern China with Different HBsAg ELISA Results by Two Kits

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Abstract

Background: The blood donors tested reactive (R) for hepatitis B surface antigen (HBsAg) but were missed by routine nucleic acid test (NAT), which can be resulted by the infection of hepatitis B virus (HBV) with extremely low viral load. This phenomenon remains a strict threat to blood transfusion in China.

Objectives: We aimed to investigate and analyze the molecular characteristics of HBV among blood donors that detected as HBsAg reactive (R) in single ELISA Reagent.

Methods: The blood donations were detected as HBsAg R in one ELISA kit, using two kinds of ELISA reagents. These samples with non-reactive (NR) results by NAT were collected and tested for HBsAg by Abbott chemiluminescent microparticle immunoassay (CLIA) with neutralization test, the level of HBsAg were further detected by Roche electrochemiluminescence immunoassay (ECLIA). The viral basic core promoter (BCP) and pre-core (PC) and S regions were also amplified by nested-PCRs. Quantitative real-time PCR (qPCR) for viral load determination and individual donation NAT of Roche reagent were adopted simultaneously. HBsAg was confirmed by the results of CLIA, ECLIA, nested-PCR, qPCR, and ID-NAT.

Results: Of 100,252 donations, 38 and 41 were identified as HBsAg reactive only in WanTai and DiaSorin ELISA kit respectively, after blood screening using WanTai and DiaSorin ELISA assays. 79 (0.077%, 79/100,252) blood samples with ELISA R-NR and NAT NR results were enrolled in the study. Of which, 17 (21.5%, 17/79) were confirmed as HBsAg positive. Of 14 cases genotyped, genotype B were 78.6% (11/14), C and D was observed in 2 (14.2%, 2/14) and 1 sample (7.1%, 1/14), respectively. Mutations in the S gene such as Y100C, Y103I, G145R, and L175S were found, which can affect the detection of HBsAg. A high-frequency mutation, T1719G (93.3%), was detected in BCP/PC and would reduce the replication of the virus.

Conclusion: A small part of blood samples with HBsAg ELISA R-NR and NAT NR results were confirmed as HBV infection, viral nucleic acids were found in most of those samples through the in-house NAT methods. It is necessary to apply more sensitive and specific assays for the detection of HBV infection among blood donors.

Introduction

According to the blood screening strategies in China, hepatitis B surface antigen (HBsAg) screening using two different ELISA reagents and nucleic acid test (NAT) were applied for HBV blood screening to ensure the safety of blood transfusion. Blood donations can be used for clinical blood transfusion as long as all the three testes give negative results. However, it has been noted that inconsistent rate between HBsAg and HBV DNA results was reported for 6%-9% among blood donations [1]. In our previous study, of 307,740 sero-negative blood samples, 80 were classified as occult HBV infection (OBI) that characterized by HBsAg non-reactive (NR) and nucleic acid test reactive (R) [2].

However, another suspicious situation that needs to be further studied is the samples with HBsAg R and HBV DNA (NAT) NR results. More specially, the samples with HBsAg ELISA R-NR and NAT NR results may have the risk of HBV transmission through blood transfusion, due to the extremely low level of HBsAg and HBV DNA. Previous studies have proved that the mutations in the S region and basic core promoter (BCP) and pre-core (PC) of hepatitis B virus can affect the detection of HBsAg and viral nucleic acid [3, 4].

In order to explore the true status of HBV infection in these blood donors, we conducted the blood screening for HBV DNA and HBsAg using ELISA, CLIA, and ECLIA. Furthermore, quantitative real-time PCR (qPCR) and nested-PCR were performed to investigate the molecular characteristics of HBV with low viral load among blood donors that detected as HBsAg reactive in single ELISA reagent, and potential cause of this special mode of HBV infection.

1. Materials And Methods

1.1 Study samples

Between February 2017 and February 2018, a total of 100,252 blood donations from Shenzhen blood center underwent HBsAg testing using two different ELISA kits (Beijing Wantai Biological Pharmacy, Beijing, China; Diasorin, Saluggia, Italy), and Procleix Ultrio Plus Assay (Grifols) (Novartis Vaccines and Diagnostics, Inc. Spain) (0.5 mL, LOD: 3.4 IU/mL) was used for the detection of HBV DNA (NAT), of which 79 (0.077%, 79/100,252) were HBsAg ELISA R-NR and NAT NR, and were enrolled in this study.

1.2 Hbsag Confirmation

All the samples with HBsAg ELISA R-NR and NAT NR results underwent HIV confirmatory algorithm: samples enrolled in the study would conduct HBsAg screening using CLIA (ARCHITECT HBsAg Reagent Kit, Abbott Ireland Diagnostics Division Finisklin Business Park, Sligo, Ireland) and Elecsys HBsAg μ (Roche Diagnostic GmbH, Mannheim, Germany) (ECLIA). Furthermore, the samples with HBsAg reactive results in CLIA would perform neutralization test using the kit of the same manufacturer (Abbott). After CLIA and ECLIA tests, those samples were further tested for HBV DNA through in-house methods (qPCR and nested-PCR) [5, 6]. Besides, individual donation NAT (ID-NAT) (Cobas TaqScreen MPX Test, version 2.0 (Roche Diagnostic GmbH, Mannheim, Germany) (1.0 mL, LOD, 2.3 IU/mL) was adopt for the detection of HBV DNA. The positive result of HBsAg was confirmed by the reactivity in CLIA and/or ECLIA, as well as any reactive results in qPCR, nested-PCR and ID-NAT [7].

The viral nucleic acid was extracted from 2.5 mL plasma using HighPure Viral Nucleic Acid Large Volume Kit (Roche Diagnostics GmbH, Mannheim, Germany) and nested-PCR was performed to amplify the BCP/PC regions (263 bp) and S-fragment (496 bp) as previously described [6]. The nested-PCR products were

purified and sequenced by Shenzhen BGI Technology Co., Ltd using Sanger methods. After DNA extraction using the nucleic acid extraction kit mentioned above, viral load of HBV was determined using qPCR [5]. The confirmation algorithm was shown in Fig. 1.

1.3 HBV phylogenetic analysis and mutations in BCP/PC and S regions

The sequences were aligned and compared with HBV reference sequences of genotype A-I (Accession numbers were shown in Fig. 2) BioEdit program. Nucleotide alignments (S region) were used to build phylogenetic tree for HBV subtyping by MEGA software using the neighbor-joining algorithm based on Kimura 2-parameter model in 1000 bootstrap replicates. The mutations in BCP/PC and S regions were analyzed and described in previous study [6]. We matched the amino acid sequences of blood donors with the reference sequences, and calculated the frequency of amino acid mutations.

1.4 Statistical Analysis

Demographic data were obtained from the donor/donation database from blood center. SPSS 21.0 software was utilized for statistical analysis.

2. Results

2.1 HBsAg confirmation and demographic characteristics of blood donors

Among 79 blood samples with HBsAg ELISA R-NR and NAT NR results in initial tests, 17 (21.5%, 17/79) were confirmed as HBsAg positive (Fig. 1). Wantai (ELISA) reported 38 reactive results (S/CO range: 1-21.89, median: 1.58) and DiaSorin (ELISA) produced 41 reactive results (S/CO range: 1-6.87, median: 1.23). Out of HBsAg positives, the median of HBsAg titer was 1.55 IU/mL, only 2 samples were below the LOD (0.05 IU/mL) of HBsAg in ECLIA. All the 17 HBsAg positive samples were NAT R. Detectable viral load were reported for 14 of the 79 (17.7%) samples, including 5 of 10–100 IU/mL, 7 of 100–1000 IU/mL, and 2 over 1000 IU/mL viral load, after qPCR test. The viral load ranged from 2649.5 IU/ml and 12 IU/ml (median, 128.5 IU/mL) (Table 1). Of the 17 HBsAg positive samples with NR results for initial NAT screening (Grifols), 14 (82.4%) and 16 (94.1%) were detected reactive in qPCR and MPX ID-NAT (Roche), respectively.

Table 1 Initial screening and confirmatory tests of HBsAg and demographic characteristics

ID	Initial Screening			Confirmatory test								Final result	D	
	NAT Grifols	ELISA-HBsAg		CLIA-HBsAg			ECLIA-HBsAg		Nested-PCR		qPCR IU/mL			Roche ID- NAT
		Wantai	Sorin	S/CO	Result	Neutralization test result	Titer	Result	BCP	S- subtype				
26	-	0.18	1.3	14.01	+	+	0.50	+	+	B	128.5	+	Positive	4
33	-	0.06	1.03	5,675.14	+	+	398.70	+	+	B	514	+	Positive	4
34	-	0.21	1.1	30.47	+	+	1.55	+	+	B	536	+	Positive	4
55	-	0.19	2.13	5.08	+	+	0.08	+	+	D	12	+	Positive	2
80	-	0.04	6.87	58.71	+	+	1.64	+	-	-	116.4	+	Positive	4
81	-	0.52	1.52	530.64	+	+	11.65	+	+	B	83.2	+	Positive	2
87	-	0.33	1.13	3.09	+	+	0.06	+	+	B	49.6	+	Positive	2
88	-	0.05	1.23	422.21	+	+	14.98	+	+	C	-	+	Positive	4
95	-	0.06	1.39	12.64	+	+	0.53	+	+	-	407	+	Positive	3
97	-	0.68	1.93	765.92	+	+	18.71	+	+	B	2654.3	+	Positive	3
219	-	0.51	1.61	1.31	+	+	<0.05	-	+	-	-	-	Positive	3
28	-	2.11	0.51	45.27	+	+	2.06	+	+	B	378.6	+	Positive	2
32	-	1.03	0.5	4.78	+	+	0.46	+	+	B	74.5	+	Positive	2
46	-	1.06	0.50	3.23	+	+	0.18	+	+	B	14.1	+	Positive	3
57	-	1.08	0.51	0.66	-	-	0.05	+	+	C	34.8	+	Positive	2
83	-	1.41	0.42	246.90	+	+	10.97	+	+	B	1373	+	Positive	2
204	-	4.13	0.45	1.23	+	+	<0.05	-	-	B	-	+	Positive	3

A majority of HBsAg positives (64.7%, 11/17) were 18–35 years old and first-blood donors (58.8%, 10/17). Nearly half of blood donors were female (47.1%) (Table 1).

2.2 Hbv Genotype Classification

S fragments of 14 samples were successfully sequenced. A phylogenetic tree of S sequence of HBV was constructed in Mega software based on Kimura-2 parameter model with 1000 bootstrap (Fig. 2). 11 (78.5%, 11/14) cases of HBV B genotype, 2 (14.3%, 2/14) cases of genotype C, and 1 (7.1%, 1/14) case of D genotype were identified among the 14 isolates.

2.3 Mutations In S And Bcp/cp Sequences

A total of 32 mutations in S sequence were observed in 13 blood donors. As shown in Table 2, 45.5% (5/11, 45.5%) S regions in 11 HBV genotype B isolates had N40S mutations. Several mutations associated with the interference with HBsAg detection, such as mutations in major hydrophilic region (MHR): Q129H, T131I, M133L/S/T, F134L, T143M, and G145R; mutations in outside of MHR: Y100C, L175S, and Y103I were found in S genes among HBV infected blood donors. In addition, “immune escape-mutant” containing Q129H, T131I/T, G145R, and E164V were also found in these S sequences.

Table 2
Mutation analysis in S and BCP/PC sequences.

Donor ID	Genotype	Mutation site		qPCR IU/mL	HBsAg test results			
		S region	BCP/PC region		Wantai ELISA	Sorin ELISA	Abbott CLIA	Roche ECLIA
26	B	Y100C, F134L, G145R		128.5	-	+	+	+
28	B	N40S		378.6	+	-	+	+
32	B	I86T, Q129H, T131I, M133S, Y161H		74.5	+	-	+	+
33	B	None		514	-	+	+	+
34	B	N40S, P46T		536	-	+	+	+
46	B	N40S, K122R, Y161F		14.1	+	-	+	+
55	D	M131I, I150M		12	-	+	+	+
57	C	G96V		34.8	+	-	-	+
80	-	-		116.4	-	+	+	+
81	B	S55F		83.2	-	+	+	+
83	B	N40S		1373	+	-	+	+
87	B	M103I, M133T		49.6	-	+	+	+
88	C	G96V		-	-	+	+	+
95	-	-		407	-	+	+	+
97	B	N40S, L49P, S55F, S58C, C64S, C76F, T143M		2654.3	-	+	+	+
204	B	S174N, K122R, E164V		-	+	-	+	-
219	-	-		-	-	+	+	-

-: Non-reactive, +: Reactive, None: no mutation.

21 mutations were identified in core upstream regulatory sequence (CURS) (nt1678-1741) from 16 samples, T1719G occurred in 93.8% (15/16) samples. The most common mutations in BCP region (nt1742-1849) and pre-C region were A1752G, A1846T. A1762T and G1764A mutations were found in 2 cases. In addition, two insertion mutations were observed in nt1847 and nt1915 (insertion of base C).

Discussion

HBV infection remains a major threat to public health and blood safety. In China, about 50% individuals have a history of HBV infection, and 7.2% are chronic carriers of HBsAg [8]. HBsAg and HBV DNA screening were implemented among blood donors to decrease the risk of HBV transmission through blood transfusion [9]. Complicated infectious mode and molecular characteristic of HBV in China can be a challenge for the detection of HBV. Notably, the samples with HBsAg ELISA R-NR and NAT NR results may have the risk of HBV transmission, due to the low level of HBsAg and HBV DNA. Due to the blood screening strategy in China, limited data about the molecular diversity and mutation analysis of HBV in blood donors are currently available. Hence, we firstly investigated the molecular characteristics of HBV among blood donors, who were missed by initial NAT screening but were detected as HBsAg ELISA R-NR, after using two different ELISA kits.

In this study, 79 samples with HBsAg ELISA R-NR and NAT NR results were found in 100,252 blood donations. After a series of confirmatory tests, of which 17 of 79 (21.9%) samples were confirmed as HBsAg positive, accounting for 0.017% (17 of 100252) of the overall sample pool (95%CI: 0.0097%-0.026%). Previous study reported that 10% blood samples with HBsAg ELISA R-NR results were transferred to ELISA R-R results after follow-up study [10], and HBV DNA can be detected in 13.3% (4/30) of such ELISA R-NR samples using qPCR method [11], which was lower than our results (21.9%). This phenomenon may result by the difference between reagent manufacturers.

17 samples were missed by one of the two ELISA kits, but most of them were detected by CLIA (16) and ECLIA (15). Currently, ELISA is the only serological assay approved for HBV blood screening in China, automated CLIA and ECLIA had been used in clinical laboratories but not applied for HBV screening among

blood donors. Furthermore, the 17 samples were tested NR by Crifols, but most of them were tested R by nested-PCR (16) and qPCR (14). Hence, more sensitive serological and NAT assays are needed for blood screening.

In addition to the different capabilities for the detection of HBV, HBV genetic diversity and various mutations of HBV strains should be taken into consideration for the inconsistent results of HBV screening assays. A majority of HBV positive blood donors in the study were genotype B (11/13, 84.6%), which was similar to our previous report in Shenzhen in 2015 (14/15, 93.3%) [8]. A nationwide survey reported that HBV genotype B was predominant in southern areas in China [12]. Genotype D mainly circulated in mediterranean region and some Asian countries [13]. One genotype D strain observed in our study reminded us the increased international travel and immigration may have contributed to the input of the epidemic.

HBV is prone to carry mutations in varying gene regions. Typically, mutations in HBsAg region can cause immune escape, resulting in the failure to detect the HBsAg [14]. Furthermore, HBV “immune escape-mutant” were highly contagious and pathogenic for immunodeficiency patients [15]. In vivo studies have found that certain mutations in the S region of HBV can lead to reduced secretion of virus particles, even prevent secretion of virus particle and reduce HBsAg synthesis [16]. Mutations in MHR and non-MHR of S region, such as Y100C, Y103I, Q129H, T131I, M133L/S/T, F134L, T143M, G145R, L175S, were observed in the study, and could reduce the affinity of monoclonal antibodies for “a” epitopes by altering the structure of HBsAg protein [17], which may contribute to the failure of the commercial reagents.

Mutations in the BCP/PC region can regulate the secretion of hepatitis e antigen (HBeAg), leading to the cessation of e antigen production. Typically, BCP mutations (A1762T and G1764A) can reduce the mRNA synthesis of pre-C region, reflected as a very low level of HBV DNA [18]. G1896A is another typical mutation in the pre-C region, which generates a stop codon at the 28th amino acid position of the HBeAg sequence, resulting in the inhibition of protein synthesis [19]. These three mutations in BCP/PC region found in this study were proved to be highly associated with the development of hepatocellular carcinoma [18]. 15 cases of T1719G mutations in BCP/PC region were identified from 16 samples in our study, which can inhibit in vitro HBV replication [20], may lead to a low viral load of HBV DNA.

It is worth noting that among the 17 samples with HBV infection, 2 samples (ID: 83, 97) had high level of viral load (> 1000 IU/mL) detected by qPCR, but reported as NR in routine NAT test (Crifols). N40S mutation in S region was identified in both samples. Whether it has an impact on NAT needs further study. Some research reported the false negative results and underestimated viral load among blood donors with HBV infection in various commercial kits [21, 22]. Therefore, NAT with two targets in different gene regions were recommended to avoid the mismatch in target regions, due to the mutations.

In summary, we analyzed the molecular characteristics of HBV among blood donors with routine ELISA R-NR and NAT NR results. Most of them were detected by CLIA and ECLIA using HBsAg reagents, and tested as R in qPCR and nested-PCR. Analysis of the S gene sequence revealed a large number of gene mutations, which have been proved to be associated with the detection of HBsAg. Some mutations in BCP/PC regions were frequently identified in this study, which may lower the viral load of HBV DNA and cause false negative NAT results. Our results reminded us the necessity to improve the sensitivity of HBsAg screening and NAT methods, especially to improve the ability to detect strains carrying mutants.

Declarations

Availability of data and materials

The data for this study is available from the corresponding author on reasonable request.

Ethics approval and consent to participate

The study was approved by the Ethics Committee in Shenzhen blood center. The methods in the study were in accordance with the guidelines of the Declaration of Helsinki. Written informed consent was obtained from all subjects participating in this research.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no conflicts of interest.

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Authors' contributions

XY designed the study. TL, RZ, RL conducted the laboratory tests. XY, HL, and JZ1 collected and analyzed data and prepared the manuscript. XY, JZ1 and JZ2 edited and reviewed the manuscript. All Authors critically reviewed and revised the manuscript drafts, approved the final version of the manuscript and take responsibility for the integrity of the data and accuracy of data analysis.

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Figures

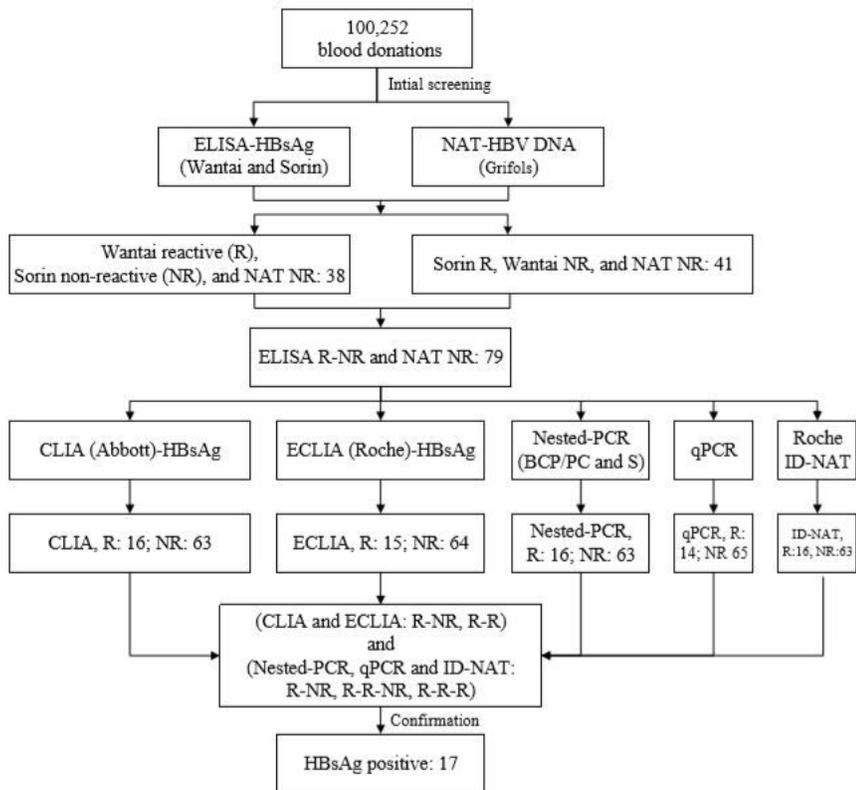


Figure 1

The study route, R: reactive, NR: non-reactive, Wantai: Diagnostic Kit for Hepatitis B Virus Surface Antigen (ELISA), Sorin: Murex HBsAg version3, NAT-Roche: Cobas TaqScreen MPX Test, version 2.0, NAT-Grifols: Procleix Ultrio Plus Assay, CLIA: ARCHITECT HBsAg Reagent Kit, ECLIA: Elecsys HBsAg $\text{\textcircled{R}}$.

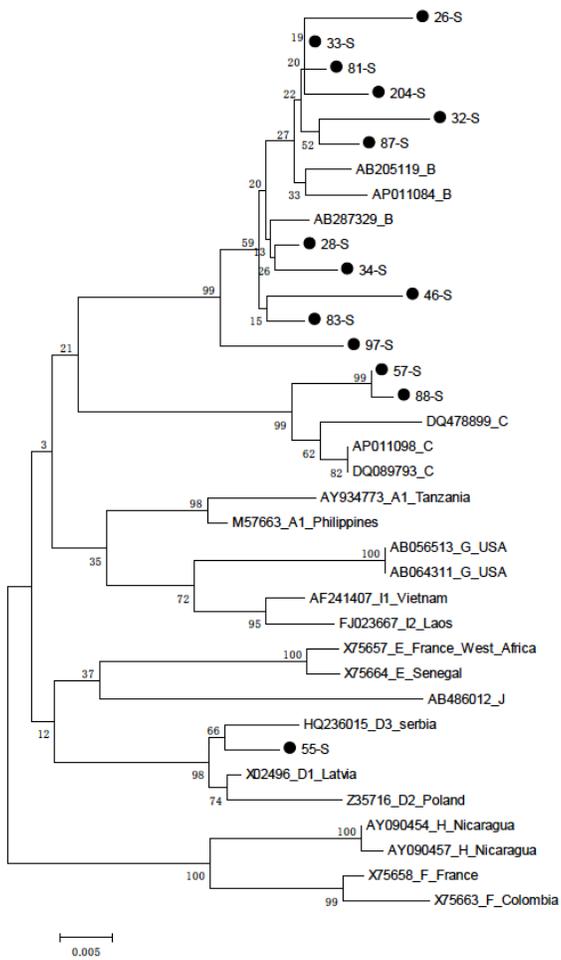


Figure 2

Phylogenetic tree analysis of S region sequences among 14 HBV isolates in blood donors.