Comparative analysis of hepatitis B virus infections in blood donors born before and after the implementation of universal HBV vaccination in southern China

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Abstract
Background: In China, the vaccinated blood donors have rapidly increased by recent years, which may impact blood safety. The true prevalence of HBV between vaccinated blood donors and non-vaccinated blood donors should be explored.

Study Design and Methods: The samples of blood donors were collected and detected for serologic markers of HBV in the Shenzhen Blood Centre (SZBC). The discrepant results were tested with commercial electrochemiluminescence immunoassay (ELCI) for HBsAg, anti-HBs, HBeAg, Anti-HBe and Anti-HBc, alternative MPX ID NAT, nested PCR, and a quantitative real-time polymerase chain reaction (qPCR) assay for HBV DNA. The serological and molecular characteristics of HBV infected blood donors were analysed, and the effects on blood safety for donors born before and after the implementation of universal HBV vaccination were compared.

Results: Out of 242 presumed HBV infected donors from 26,318 donations, 131 (0.49%, [95% CI, 0.43–0.59]) chronic HBV infections (CHB, HBsAg detected with or without DNA), 58 (0.22%, [95% CI, 0.17–0.28]) occult hepatitis B infections (OBI, HBsAg not detected, assume anti-HBc positive and/or anti-HBs with HBV DNA) and 3 (0.011%, [95% CI, 0.0023–0.033]) window period (WP) infections were confirmed respectively. There were 28 CHBs (0.44%), 7 OBIs (0.11%) and 1 WP (0.016%) from vaccinated blood donor and 103 CHBs (0.52%), 51 OBIs (0.26%) and 2 WPs (0.01%) from non-vaccinated blood donor. The HBV+ (CHBs, OBIs and WPs) rate (0.56%) in vaccinated donors was lower than in non-vaccinated donors (0.78%, p < 0.05). The HBsAg titers of vaccinated infected blood donors (Median: 128.8 IU/ml) were much higher than non-vaccinated infected blood donors (58.4 IU/ml). The OBI yield rates in the vaccinated blood donors was significantly lower than the non-vaccinated blood donors (p < 0.05). There 102/124 (82.3%) samples were genotype B, 22/124 (17.7%) were genotype C respectively. There was no significant difference in the distribution of genotype between non-vaccinated blood donors (B/C, 86/17) and vaccinated blood donors (B/C, 23/6; p > 0.05). High

Abbreviations: BCP/PC, basic core promoter/pre-core; CHB, chronic hepatitis B; CTL, cytotoxic CD8+ lymphocytes; dHBV, discriminatory procleix ultrio plus test identifying HBV DNA; ECLI, electrochemiluminescence immunoassay; ELISA(s), enzyme-linked immuno-absorbent assay(s); HBIG, hepatitis B immunoglobulin; HBV, hepatitis B virus; ID, individual donation; IR, initial reactive; LOD, limit of detection; MHC, major histocompatibility complex; MHR, major hydrophilic region; NAT, nucleic acid testing; NRR, non-repeat reactive; OBI, occult hepatitis B infection; S/CO, sample to cutoff; TMA, transcription-mediated amplification.
frequency of vaccine escape mutations M133L (32.4%) and E164G in S region of genotype B strains and substitution L175S (40.9%) related to vaccine escape in S region of genotype C strains were identified.

**Conclusion:** The universal HBV vaccination program markedly reduces the risk of HBV infection in blood donors, and provides a significant guarantee for the safety of blood transfusion. Several important mutations detected related vaccine escape and notable mutations needed further investigated.

**KEYWORDS**

blood donors, hepatitis B virus, mutations, the universal HBV vaccination program

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**1 | INTRODUCTION**

HBV infection continues to be a major public health concern worldwide despite the availability of an effective vaccine and potent antiviral treatments. One-third of the world’s population is predicted to have been infected by HBV. The WHO estimates that 257 million people are currently living with a HBV infection and approximately 885,000 deaths were cause by HBV-associated complications in 2015. In China, nearly 50% of the Chinese population has a history of HBV infection.

To curb hepatitis B epidemic, the universal HBV vaccination has been implemented by Chinese government from 1992 and resulted in HBsAg carrier reduction significant from 10% to <1% in children group over two decades. These vaccinated populations have enrolled in blood donors since 2010. HBV vaccines were gradually becoming the majority of blood donors in China and impacting blood safety positively. In our previous studies for exploring the true HBV infection in blood donors, hepatitis B virus, mutations, the universal HBV vaccination program markedly reduces the risk of HBV infection in blood donors, and provides a significant guarantee for the safety of blood transfusion. Several important mutations detected related vaccine escape and notable mutations needed further investigated.

**KEYWORDS**

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**2 | MATERIAL AND METHODS**

A total of 26,318 blood donors were enrolled in this study from Jan 2016 to Jun 2016. The presumed vaccinated and non-vaccinated blood donor populations were separated into two groups, according to being born before or after 1 Jan, 1992. Blood donors born after 1 Jan, 1992, were designated as vaccinated donors. Blood donors born before 1 Jan, 1992, were selected as non-vaccinated donors. There were 6421 (24.4%) donors for vaccinated groups and 19,897 (75.6%) donors for the non-vaccinated groups. The presumed vaccinated and non-vaccinated donors were included in this study. The pre-donation questionnaire, rapid pre-donation testing and dual ELISA assays for routine screening are described as previous study. Blood samples with HBsAg reactivity were re-tested in duplicate using at least one ELISA assay. Samples were determined to be HBsAg ELISA- if results were reactive in any assay in re-testing. The donors were defined as first-time blood donors who gave blood for the first time, while donors were defined as repeat-donors who donated blood more than once at the SZBC. Multiple Procleix ultrio plus assay (Grifols diagnostic solutions, Inc. and Roche) was used to detect HBV (limit of detection (LOD): 3.4 IU/ml), HCV and HIV-1 genomes in all donors. Individual reactive samples were further tested with a discriminatory Procleix Ultrio plus test to identify the virus responsible for NAT reactivity (HBV, HCV, or HIV-1) as the manufacturer recommends. If necessary, MPX2.0 ID HBV NAT (LOD: 2.3 IU/ml) was used as an alternative HBV NAT assay for further identification. Serum and aliquots of the index-retrieved frozen plasma unit for HBsAg ELISA- and/ or NAT initial reactive were collected for additional determination.

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**3 | SUBJECTS STUDIED**

**3.1 | Supplemental serological testing, HBsAg quantitation, and HBsAg+ confirmation**

For all HBsAg ELISA- and/ or NAT initial reactive samples, HBsAg (LOD: 0.05 IU/ml) and anti-HBs (LOD: 2 IU/L) were quantified, hepatitis B e antigen (HBeAg), anti-HBe, and hepatitis B core antibodies (anti-HBc) of were tested by commercially available ECLI (Roche). Any sample testing repeats reactive/ positive by supplemental HBsAg assays and HBV DNA+ was considered HBsAg confirmed positive (HBsAg+).
3.2 | HBV DNA confirmation

A 200–2500 μl HBsAg ELISA + and / or NAT initial reactive samples were extracted for HBV DNA by HighPure Viral Nucleic Acid Large Volume Kits (Roche Diagnostics Gmbh, Mannheim, Germany). The basic core promoter / pre-core (BCP / PC) and S region were amplified by qPCR (LOD:5 IU/ml) and nested PCRs (LOD:10 IU/ml) for further detection. As mentioned above, the samples reacted by any two of the five NAT methods were confirmed to be DNA positive. Donors that tested HBsAg + and HBV DNA + with anti-HBc + were designed as chronic hepatitis infections (CHB), donors that tested HBsAg- and HBV DNA + with anti-HBc + and/ or anti-HBs + were OBIs, and some donors with HBsAg-, anti-HBc-, anti-HBs- but HBV DNA + were designed as serological window period (WP).6

3.3 | HBV DNA sequencing, genotyping and comparison

To confirm HBV genotypes, the amplified PCR products obtained from the BCP/PC (295 bp) and the S regions (495 bp) were sent to Shanghai Invitrogen Co., Ltd. (Guangzhou, China) for sequencing. HBV genotype determination was performed by phylogenetic analysis using the MEGA7.0 program. The neighbour-joining method based on Kimura 2-parameter mode and complete deletion for gaps with 1000 bootstrap replications was used. Bootstrap values of 70% or greater were considered significant. Amino acid sequences isolated from HBsAg+ blood donors from China, Thailand and Malaysia were used as reference sequences.12

3.4 | Statistical analyses

The 95% confidence interval (95% CI) of the observed rate was calculated by binomial exact proportion method. Fisher’s exact test was used to compare categorical variables. For continuous variables, non-parametric Mann Whitney test was used, with p value <0.05 as the cut-off value of significance.

4 | RESULTS

4.1 | Demographic characteristics and screening results of blood donors samples

There 6421 vaccinated and 19 897 non-vaccinated blood donors were enrolled in this study, the demographic characteristics of vaccinated and non-vaccinated donors were shown in Table 1. After screening by dual ELISAs and NAT, 154 (0.59%) were HBsAg ELISA+.

| TABLE 1 Demographic characteristics of vaccinated and non-vaccinated donors |
| --- | --- | --- | --- |
| Donors status | Overall population (%) | Vaccinated group (%) | Non-vaccinated group (%) | p |
| First-time | 14 738 (56.0) | 4810 (74.9) | 9929 (49.9) | 0.00 |
| Repeat | 11 580 (44.0) | 1611 (25.1) | 9968 (50.1) | 0.00 |
| Gender | | | | 0.00 |
| Male | 16 975 (64.5) | 3501 (54.5) | 13 475 (67.7) | |
| Female | 9343 (35.5) | 2920 (45.5) | 6422 (32.3) |
| Occupation | | | | 0.00 |
| Farmers | 482 (18.2) | 28 (0.4) | 454 (2.3) | |
| Workers | 4173 (15.8) | 472 (7.3) | 3701 (18.6) |
| Students | 1778 (6.7) | 1653 (25.7) | 125 (0.6) |
| Soldiers | 150 (0.5) | 76 (1.2) | 74 (0.4) |
| Teachers | 287 (1.1) | 79 (1.2) | 208 (1.0) |
| Civil servants | 255 (1.0) | 26 (0.4) | 229 (1.1) |
| Doctors | 369 (1.3) | 101 (1.6) | 268 (1.3) |
| Staff | 9920 (37.7) | 1797 (28.0) | 8123 (40.8) |
| Others | 8904 (33.8) | 2189 (34.1) | 6715 (33.7) |
| Education | | | | 0.00 |
| Below high school | 5405 (20.5) | 1290 (20.1) | 4115 (20.7) |
| High school and Assonate degree | 15 565 (59.1) | 3970 (61.8) | 11 595 (58.2) |
| Bachelor’s degree | 4852 (18.4) | 1131 (17.6) | 3721 (18.7) |
| Master’s degree | 338 (1.3) | 22 (0.3) | 316 (1.5) |
| Others | 158 (0.6) | 8 (0.1) | 150 (0.8) |
| Total | 26 318 (100) | 6421 (24.4) | 19 897 (75.6) |
and 26,164 (99.41%) were HBsAg ELISA-. Of 154 HBsAg ELISA+ samples, 113 (0.43%) were NAT initial reactive (IR), and 41 (0.16%) were NAT-. Of these 26,164 HBsAg ELISA- samples, there were 88 NAT IR; 34 of 88 samples were positive for discriminatory Procleix Ultrio plus test identifying HBV DNA (dHBV), and 54 of 88 were multiplex NAT reactive, but negative for discriminatory HBV/HCV/HIV...

FIGURE 1 Flowchart for confirmatory testing algorithm of HBsAg ELISA+ and/or NAT IR samples
test (non-repeat reactive, NRR). A total of 26,076 (99%) samples were HBsAg ELISA- and NAT- (see Figure 1).

### 4.2 Confirmatory results of HBsAg ELISA+ samples and HBV DNA NAT IR samples

A total of 41 (of 154) HBsAg ELISA+ samples were retest by HBsAg ECLI and 4 alternative NATs, 18/41 (43.9%) were HBsAg ECLI+ and alternative NATs+, and the remaining 23 samples were HBsAg ECLI- and NATs-. Of 154 HBsAg ELISA positive samples, there 131 (85.1%) cases were confirmed HBsAg+ by ECLI and DNA+ by NATs, 23 confirmed HBsAg- by ECLI and DNA- by NATs. (See Figure 1).

A total of 34 HBsAg ELISA-/dHBV+ samples were re-tested by nested PCRs, qPCR and MPX ID HBV NAT, 31 (of 34) were confirmed HBV DNA+. Of 54 HBsAg ELISA-/NAT NRR samples, 30 (55.6%) were confirmed DNA+. In total, 192 (0.74%) confirmed HBV+ (see Figure 1).

### 4.3 Statistical result of demographic characteristics and testing results

Successful HBV S gene sequencing followed by genotyping was performed for 124 of the 192 samples, which composed the vaccinated group and non-vaccinated group. The results showed statistically significant differences (p < 0.05) in sex, number of first time and repeated time donors, occupation distribution, education distribution, the rate of HBV+, mean HBsAg titers, Anti-HBc+/Anti-HBs+ pattern, and OBI yield rate, the distribution of anti-HBs titers between the two groups (Table 1, Table 2); however, no significant differences in the distribution of HBV genotype, and the median of anti-HBs titers between the two groups were found (p > 0.05).

### 4.4 Prevalence of HBV, HBsAg and OBI between blood donors born before and after the universal infant vaccination program

A 192/26318 (0.73%, [95% CI, 0.61–0.82]) HBV+, 131/26318 (0.50%, [95% CI, 0.41–0.58]) HBsAg+, 58/26318 (0.22%, [95% CI, 0.16–0.28]) OBI were detected and verified in the eligible blood donors population respectively. Of 192 HBV+ blood donors, 36 were vaccinated donors and 154 were non-vaccinated donors, The rate of HBV+ in the vaccinated donors is lower than in the non-vaccinated donors (p < 0.05). Of 131 HBsAg confirmed positive donors, 28 (0.44%) were vaccinated donors, and 103 (0.52%) were non-vaccinated donors. Of 58 cases OBIs, 7 (0.11%) were vaccinated donors, and 50 (0.26%) were non-vaccinated donors. There is a significant difference between the two groups (p < 0.05).

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**TABLE 2** Classification of 192 infected blood donors between vaccinated and non-vaccinated groups

| Overall population | Vaccinated group | Non-vaccinated group | p     |
|--------------------|------------------|----------------------|-------|
| HBV+ (%)           | 192 (0.73)       | 36 (0.56)            | 156 (0.78) | 0.040 |
| HBsAg+/DNA+ (%)    | 131 (0.50)       | 28 (0.44)            | 103 (0.52) | 0.229 |
| HBsAg titers median (IU/ml) | 82.5          | 128.8                | 58.4    | 0.002 |
| Min.               | 0.06             | 0.06                 | 0.06    |       |
| Max.               | 919              | 919                  | 650.2   |       |
| HBsAg-/DNA+ (%)    | 61+ (0.22)       | 8 (0.12)             | 53 (0.26) | 0.031 |
| Anti-HBc-/Anti-HBs+ (%) | 3 (0.011)       | 0 (0.00)             | 3 (0.015) | 0.432 |
| Anti-HBc+/Anti-HBs- (%) | 25 (0.095)     | 4 (0.052)            | 21 (0.11) | 0.234 |
| Anti-HBc-/Anti-HBs+ (%) | 30 (0.11)       | 3 (0.048)            | 27 (0.14) | 0.044 |
| Anti-HBc+/Anti-HBs- (%) | 3 (0.011)       | 1 (0.016)            | 2 (0.010) | 0.568 |
| OBIs               | 58 (0.22)        | 7 (0.11)             | 51 (0.26) | 0.04  |
| WPs                | 3 (0.11)         | 1 (0.16)             | 2 (0.10)  | 0.568 |
| Anti-HBs titre     |                  |                      |        |
| <10 IU/L (negative) | 152              | 31                   | 121     |       |
| 10–100 IU/L        | 27               | 5                    | 22      |       |
| >100 IU/L          | 13               | 0                    | 13      |       |
| Median (IU/L)      | <2               | <2                   | <2      | 0.205 |
| Min.               | <2               | <2                   | <2      |       |
| Max.               | >1000            | 91.7                 | >1000   |       |
| Genotype B (%)     | 102 (0.38)       | 23 (0.36)            | 79 (0.40) | 0.254 |
| Genotype C (%)     | 22 (0.099)       | 6 (0.093)            | 16 (0.075) | 0.501 |

*2 WP and 57 OBI. HBsAg < 0.05 IU/ml: Negative. Anti-HBs < 10 IU/L: Negative.

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| Genotype | Infections | Group     | Mutation  | Frequency (%) | Vaccine escape mutant | Affect serological diagnosis | Failure in HBlg therapy | References |
|----------|------------|-----------|-----------|---------------|-----------------------|-------------------------------|--------------------------|------------|
| B        | CHB        | Non-vaccinated | N40S/F    | (25 ± 1)/65(40) | This study             | This study                   | This study             | This study |
|          |            |           | I68T/M    | (2 ± 2)/65(6.2) | This study             | This study                   | This study             |            |
|          |            |           | Q101R/H/K | (2 ± 1)/65(6.2) | This study             | Yes (Q101K)                  | This study             |            |
|          |            |           | M133L/T/S | (11 ± 5)/2/65(27.7) | Yes (M133L,M133T)     | Yes (M133L,M133T)           | Yes (M133L)           | 13,15      |
|          |            |           | F134I/L/S | (3 ± 1)/65(7.8) | This study             | Yes (F134I)                 | This study             |            |
|          | Vaccinated | G44D/E    |           | (8 ± 1)/2/65(38.1) | This study             | This study                   | This study             |            |
|          |            |           | M133L     | 11/2(52.4) | Yes (M133L,M133T)     | Yes (M133L,M133T)           | Yes (M133L)           | 14,15      |
|          |            |           | E164G     | 3/2/14(43) | Yes (E164G)           | Yes (E164G)                 | This study             |            |
| OBI      | Non-vaccinated | G44D/E    |           | (5 ± 1)/14(42.9) | This study             | This study                   | This study             |            |
|          |            |           | M133L     | 9/14(64.3) | Yes (M133L,M133T)     | Yes (M133L,M133T)           | Yes (M133L)           | 14,15      |
|          | Vaccinated | K24R      |           | 2/100       | This study             | This study                   | This study             |            |
|          |            |           | G44D      | 2/2(100)   | This study             | This study                   | This study             |            |
|          |            |           | M133L     | 2/2(100)   | Yes (M133L,M133T)     | Yes (M133L,M133T)           | Yes (M133L)           | 14,15      |
| C        | CHB        | Non-vaccinated | Q30K      | 3/5(60)      | This study             | This study                   | This study             | This study |
|          |            |           | S34L      | 3/5(60)      | This study             | Yes (S34L)                  | This study             | 17         |
|          |            |           | N40S      | 4/5(80)      | This study             | This study                   | This study             |            |
|          |            |           | A45T      | 3/5(60)      | Yes (A45T)            | This study                   | This study             |            |
|          |            |           | T47V      | 3/5(60)      | a                     | This study                   | This study             |            |
|          |            |           | P49H      | 3/5(60)      | This study             | This study                   | This study             |            |
|          |            |           | S55F      | 3/5(60)      | This study             | This study                   | This study             |            |
|          |            |           | I68T      | 3/5(60)      | This study             | This study                   | This study             |            |
|          |            |           | P79H      | 3/5(60)      | This study             | This study                   | This study             |            |
|          |            |           | L175S     | 3/5(60)      | a                     | This study                   | Yes (L175S)           | 19         |
|          |            |           | V177A     | 3/5(60)      | This study             | Yes (V177A)                 | This study             | 19         |
|          | Vaccinated | T118K     | 1/3(33.3)  | Yes (T118R)  | This study             | Yes (T118R)                | Yes (T118R)           | 13         |
|          |            |           | L175S     | 1/3(33.3)    | a                     | Yes (L175S)                 | This study             | 19         |
| OBI      | Non-vaccinated | Q30K      | 4/11(36.4) | This study             | This study                   | This study             | This study             |            |
|          |            |           | N40S      | 5/11(45.5)   | This study             | This study                   | This study             |            |
|          |            |           | A45T      | 5/11(45.5)   | This study             | This study                   | This study             |            |
|          |            |           | T47V/K    | (5 ± 1)/11(54.5) | a                   | This study                   | This study             |            |
|          |            |           | P49H/L    | (4 ± 1)/11(45.5) | This study             | This study                   | This study             |            |
|          |            |           | S55F      | 4/11(36.4)   | This study             | This study                   | This study             |            |
|          |            |           | R59H      | 4/11(36.4)   | This study             | This study                   | This study             |            |
4.5 | S gene sequencing and phylogenetic analysis

A total of 242 samples were amplified by nested PCR, 124 cases were got s sequences. In the non-vaccinated group, 95 were got s sequences, 79 (83.1%) were genotype B, 16 (16.9%) were genotype C. And in the vaccinated group, 29 samples were got s sequences, 23 (79.3%) were genotype B, 6 (19.7%) were genotype C respectively. There is no difference between the two groups for genotype distribution ($p = 0.790 > 0.05$).

4.6 | Mutation analysis within or out of MHR in identified HBV+ donations

S sequences including the major hydrophilic region (MHR) from 124 samples (non-vaccinated group: 79 genotype B and 16 genotype C strains; vaccinated group: 23 genotype B and 6 genotype C strains) were analysed. Compared with reference amino acids, the occurrence of the donations’ amino acid substitutions at each position observed with significant difference ($p < 0.05$) were determined as notable mutations.$^7$ (Table 3). For the amino acid sequence encoded by the S gene from 65 CHBs genotype B samples in non-vaccinated group, N40S/F (40%), I68T/M (6.2%), Q101R/H/K (6.2%), M133L/T/S (27.7%) and F134I/l/S (7.8%) were calculated as notable mutations, and 224 amino-acid substitutions were presented in this group. However, in vaccinated group (CHB), G44D/E (38.1%), M133L (52.4%) and E164G (14.3%) were notable mutations, the frequency of M133L (47.6%) mutation were higher than in non-vaccinated group (CHB, 16.9%, $p < 0.05$). In non-vaccinated OBI group for genotype B, only G44D/E (42.9%) and M133L (64.3%) were observed as notable mutations. However, in vaccinated OBI group, all were found K24R, G44D and M133L mutations. Meanwhile, in genotype C donations, Q30K, S34L, N40S, A45T, T47V, P49H, F55S, I68T, P79H, L175S, V177A were notable mutations in non-vaccinated CHB group. T118K, L175S notable mutations were found in vaccinated CHB group. While 11 OBIs (non-vaccinated group), Q30K, N40S, A45T, T47V/K, P49H/L, S55F, R59H, T113P/S/N, T118K/R, S143T/L, R160K/N, L175S and V177A were determined as notable mutations. G145R, G145A, G145E were observed in 1 genotype C vaccinated CHB donation, and 1 non-vaccinated CHB donation respectively.

5 | DISCUSSION

China is endemic for hepatitis B, and the residual risk of transfusion transmission is significantly higher for HBV (1:17501) than for HIV-1 (1:903498) and HCV (1:59588).$^{22}$ HBV NAT has been preliminarily introduced in some major blood centres in China since 2003, and the HBV NAT yield ranged between 1:1000 to 1:10000.$^{23}$ In China, neo-natal vaccination resulted in a decrease of HBV incidence.$^5$ However, vaccination may also favour the development of escape mutants and neutralising anti-HBs antibodies level decrease over time in vaccinated people who may become susceptible to HBV infection.$^{24}$ In our
study, algorithms of confirmatory testing and supplemental testings are adopted to avoid false-positive results and identified the low-virus-load donations utmostly as many investigations. Confirmed positivity was based on multiple assay reactivity or sequences generated as accurate as possible. These measurements would give a better comparison between vaccinated blood donors and non-vaccinated blood donors for the prevalence of HBV, HBsAg and OBI. In this study, 242 reactive by NAT and/or HBsAg ELISA samples from 26318 candidate blood donors were investigated, 23 initial HBsAg-positive and 27 HBV DNA results could not be confirmed by further testing. In total, of 192 confirmed HBV infected blood donors, 61 (31.3%) cases were HBsAg-/DNA−, and 131 (60.1%) were HBsAg+/DNA+ due to adoption of large volume DNA extraction assay. Therefore, the confirmed HBsAg positive rate is 0.50%, which coincides with the report. Of 54 NRR samples, 30 donations were clarified HBsAg+/DNA+, a higher NAT yield rate was confirmed compared with the multi-regional study and Hongkong study.

The true interdiction HBV DNA positive rate by Ultrio Plus ID-NAT screening in combination with HBsAg was 0.73%, higher than true HBsAg positive rate (0.50%, \( p = 0.001 \)), and the true OBI yield was 1:453 (58/26318), nearly had two-fold increase compared with a previous study in Shenzhen due to application of ID NAT. For The Ultrio Plus assay has used a target enhancer reagent, which helps to disrupt viral particles and exposes more single-stranded DNA for the capture probe. This assay modification increased the proportion of OBI yield at least two fold more than the Ultrio assay. Moreover, in the NAT NRR donations, 31.5% were got sequences, and 38% were qPCR positive due to shorter length of the primer, overall, 55% were identified HBV DNA positive in which 100% were low-virus-load OBI, gained another half of OBI cases. In our study with those reported in other regions, the differences of the rates varied considerably depending on HBV epidemiology, the proportion of repeat or the first time donors, NAT sensitivity, and pooling strategy used; for example, 1:624 in Xiamen China, 1:3471 in Hongkong in China, 1:894 in Taiwan in China, 1:4232 in Thailand and 1:770000 in Germany.

During the 5 months of study, HBV prevalence in vaccinated donors aged 18–24 years was lower than in non-vaccinated donors aged 25–60 (\( p < 0.05 \)). Although there was no difference in genotype distribution between OBI infected vaccinated and non-vaccinated individuals, OBI yield rates were also confirmed lower than in non-vaccinated donors (\( p < 0.05 \)). This difference might be related to the increasing cumulative HBV exposure with the ages, and vaccine-related protection would be the definite cause. The OBIds with anti-HBs in present study suggested that OBIds occur primarily in individuals who have recovered from the infection but are unable to develop an effective immune control. Furthermore, among OBI samples, the percentage of those carrying anti-HBs in vaccinated OBI blood donors is lower than the non-vaccinated OBI blood donors (\( p < 0.05 \)), suggested that lower level or less of anti-HBs were insufficiently protected and are susceptible to infection associated with breakthrough or occult HBV infections, even when vaccinated at birth. Interestingly, in HBsAg positive vaccinated blood donors, the titers are much higher than non-vaccinated blood donors (\( p < 0.005 \)), because the infected vaccinated blood donors are no response or warning off vaccines, HBsAg is secreted more than in usual after infection.

HBV with HBsAg escape mutants are rare but potentially highly infectious and pathogenic, particularly in immune-compromised recipients. The prevalence of the well-known neutralisation escape mutation G145R in the HBV envelope protein was as high as 22% in American blood donors. However, in our study, the G145R mutation was not found in genotype B; only three cases harboured G145A/E/R individually were found in genotype C donations. Furthermore, compared to G145R mutation, several mutations within and out of MHR occurred at high frequency such as M134L and L175S. Surprisingly, the isolates from the CHBs non-vaccinated genotype B group also showed high variability in their S gene sequences in comparison with CHBs vaccinated genotype B group. However, the frequency of M133L (52.4% vs. 16.9%) and E164G (14.3% vs. 3.1%) associated with escape from vaccine-induced immunity was observed higher significantly in CHBs vaccinated group than in the CHBs non-vaccinated group (\( p < 0.05 \)). This is because antibodies induced by the current vaccine may not recognise changes in the surface antigen as a result of mutation. In the genotype C non-vaccinated group, lots of mutations out of MHR such as Q34K, N40S, T47V, P49H, S55F, L175S, V177A were detected. These mutations are associated with major histocompatibility complex (MHC) class I-restricted cytotoxic CD8+ lymphocytes (CTLs) epitopes, and it has been experimentally proved that adaptive immune response mediated by CTLs is necessary for controlling HBV infection. This may be because mutations in CTL epitopes can evade cellular immunity and contribute to persistence, and are potentially responsible for vaccine breakthrough infection and HBsAg undetectability. Interestingly, two membrane-embedded C-terminus mutations L175S, V177A were observed at high frequency in genotype C donors in the present study and proved tightly to correlate with OBI, and powerfully to affect HBsAg detection.

In conclusion, the prevalence of HBV+ and OBI in vaccinated donors is lower than in non-vaccinated donors (\( p < 0.05 \)), suggested that the universal HBV vaccination program markedly reduces the risk of HBV infection in blood donors. Furthermore, there is a high frequency of mutations in the MHR and out of MHR of the HBV S gene, which may cause vaccine escape, diagnosis failure and failure in HB1g therapy problem and highlights the need for more studies into the prevalence of mutants.

**AUTHOR CONTRIBUTIONS**

Xianlin Ye designed the experiments and wrote and reviewed the manuscript. Ling Li reviewed, revised and edited the manuscript. Tong Li, Yi Li, Jinfeng Zeng, Ran Li, Xiaoxuan Xu, Xiaoyu Guan participated in the study design, performed the experiments, and collected and analysed the data. All authors read and approved the final manuscript.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data sets used and/or analysed during the current study are available from the corresponding author on reasonable request.

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