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HBsAg and anti-HBs coexistence in patients with HBV in acute and chronic phases

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ABSTRACT

HBsAg and anti-HBs coexistence represents an unusual serological pattern in hepatitis B virus (HBV) infection. However, its natural course remains unclear. This study investigated the occurrence of this serological pattern in patients with HBV in acute and chronic phases and estimated the associated risks. Of the 215 adult patients diagnosed with acute-phase HBV, 19 (8.84 %) cases demonstrated HBsAg and anti-HBs coexistence. In the chronic phase, 54 new cases of HBsAg and anti-HBs coexistence were identified during a median follow-up of 14 months (interquartile range: 14–28) among 4593 HBsAg-positive patients. The average annual incidence of coexistence was 1.41 % ± 0.28 %. The cumulative risk of HBsAg and anti-HBs coexistence in chronic phase patients was higher in those aged ≥ 50 years (risk ratio [RR]: 1.79, 95 % confidence interval [CI]: 1.04–3.09, $P = 0.035$), with positive HBeAg (RR: 3.43, 95 % CI: 1.91–6.19, $P < 0.001$), baseline alanine transaminase abnormalities (RR: 3.62, 95 % CI: 1.93–6.79, $P < 0.001$), and higher HBV DNA levels (RR: 1.97, 95 % CI: 1.12–3.49, $P = 0.017$). The quasispecies heterogeneity of the "a" determinant mutation demonstrated no significant change during the occurrence of coexistence with HBsAg and anti-HBs in HBV infection. Therefore, HBsAg and anti-HBs coexistence may be the intermediate process in the natural history of HBV infection, unrelated to "a" determinant mutation but associated with the disease phase.

1. Introduction

Hepatitis B is a significant global health concern, with over 240 million individuals chronically infected with hepatitis B virus (HBV) worldwide (Tang et al., 2018). The interrelationship between viral replication and the host immune response determines the natural course of HBV infection. Hepatitis B virus serological markers serve as biological indicators of viral replication status and prognosis. The dynamics of viral protein expression and antibody production may differ during the natural course of infection.

The coexistence of HBsAg and anti-HBs is an unusual serological finding (Kwak et al., 2019) as it includes components indicative of immunity and active infection (Lee et al., 2020). A cross-sectional observational study of patients who are HBsAg positive reported a prevalence of HBsAg and anti-HBs coexistence ranging from 1.23 % to 7.09 % (Kwak et al., 2019; Lee et al., 2020; Liu et al., 2016). The coexistence of HBsAg and anti-HBs has been associated with advanced fibrosis and hepatocellular carcinoma (Jin et al., 2019; Wang et al., 2022). Previous studies revealed that viral genome mutations, immune status, and host genetic factors may contribute to this phenomenon (Kwak et al., 2019). However, the natural occurrence of HBsAg and anti-HBs coexistence remains poorly understood.

Hepatitis B virus causes either acute, self-limited infection or acute infection that progresses to chronic disease. In this study, we investigated the natural occurrence of HBsAg and anti-HBs coexistence in patients during acute and chronic phases and estimated the relative risks associated with this occurrence.

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2. Materials AND methods

2.1. Patients

This study recruited adult patients with HBV in the acute phase from October 2013 to January 2016 through the notifiable infectious disease and emergent public health event reporting system in Wuwei People's Hospital, Wuwei Hospital of Traditional Chinese Medicine, Wuwei Cancer Hospital, Liangzhou District People's Hospital, Liangzhou District Hospital of Traditional Chinese Medicine, and Liangzhou District Third People's Hospital of Gansu province. New HBV infection was defined as HBV serum markers tested within 1 year, with the first test indicating HBsAg negative and the second test confirming HBsAg positive and anti-HBc IgM positive. If HBsAg was negative within 6 months, if anti-HBc IgM exceeded 1:1000, or if liver histology demonstrated acute viral hepatitis changes, the patient was classified as having HBV acute infection (Jindal et al., 2013). Patients in the chronic phase were recruited from a seroepidemiological survey conducted in Wuwei City, Gansu province, from June 2010 to December 2011 (Pu et al., 2016; Ji et al., 2014). Chronic HBV infection diagnosis was based on HBsAg positivity for $>$ 6 months. The occurrence of HBsAg and anti-HBs coexistence was defined as positive anti-HBs $\geq$ 10 IU/L. Chronic phase patients were followed up annually from 2012 to 2015. Patients coinfected with hepatitis C virus or human immunodeficiency virus, or those with other hepatic diseases, were excluded from the study.

2.2. Serological markers and quantitation of hepatitis B virus

Routine serological tests for HBsAg, anti-HBs, HBeAg, anti-HBe, anti-HBc IgM, and anti-HBc IgG were conducted using commercially available standard enzyme-linked immunosorbent assay (ELISA, Wantai, Beijing, China). HBsAg and anti-HBs confirmatory tests were conducted using the Roche Elecsys 2010 system with the Elecsys HBsAg assay and Elecscy Anti-HBs assay (Roche Diagnostics, USA) for carriers exhibiting HBsAg and anti-HBs coexistence. The lower detection limit of the HBsAg assay for PEI standards ad and ay was $\leq$ 0.04 U/mL, and for WHO standard ad, it was $\leq$ 0.1 IU/mL. Automatic Biochemistry Analyzers from Biobase Biodustry Corporation (Jinan, Shandong, China) were used to determine serum alanine aminotransferase levels. Serum HBV DNA levels were quantified using a fluorescence quantitative polymerase chain reaction (PCR) diagnostic kit from Daan Gene Diagnostic Center (Guangzhou, China) with a detection threshold of 10^2 IU/mL. An ABI FAST 7500 real-time PCR instrument (Applied Biosystems, Foster, CA, USA) was used to analyze the results.

2.3. Ultra-deep sequencing of the hepatitis B virus S region

Consecutive samples were collected from six chronic patients with anti-HBs production to study the quasispecies during anti-HBs dynamics. Hepatitis B virus DNA was extracted from 200 μL of serum using the TIANamp Virus DNA/RNA Kit (TIANGEN Biotech, Beijing, China), with 30 μL of RNase-free ddH_2O. Polymerase chain reaction-barcoded primers were designed to systematically assess the diversity of the HBsAg major hydrophilic region (MHR) at the nucleotide level. The HBV S gene was amplified targeting a 584 bp fragment (aa21–aa204), enabling amplification and ultra-deep sequencing of the complete "a" determinant region (aa123–aa149). The barcoded-modified primers were 5′-GCGGGGTTTTTCTTGTTGAC-3′ (nt203–nt222, forward) and 5′-GGGACTCAAGATGTTGTACAG-3′ (nt787–nt767, reverse). Polymerase chain reaction was performed using the following system: 5 μL of 5 × reaction buffer, 5 μL of 5 × GC buffer, 5 μL of dNTP (100 Mm), 1 μL of forward primer (10 μM), 1 μL of reverse primer (10 μM), 2 μL of DNA template, 6 μL of ddH_2O, under the conditions: Initial denaturation at 98 °C for 2 min, denaturation at 98 °C for 15 s, annealing at 55 °C for 30 s, extension at 72 °C for 30 s, final extension at 72 °C for 5 min, and hold at 10 °C, for 25–30 cycles. The 454 GS FLX + System was then used to sequence the amplicon.

2.4. Major hydrophilic region variants analysis

Sequences after 454-pyrosequencing were demultiplexed and quantified using the tool by Roche. Sequencing reads $<$ 250 bp were removed. A phred quality score of $>$ 20 on both strands was used for variant calling. All reads were compared to reference strains (70 % homology), and BLAST software (version 2.2.29) was used to determine orientation. After filtering, sequences were clustered and aligned against reference sequences from Genbank (www.ncbi.nlm.nih.gov/projects/genotyping/). Mutations were defined as amino acid substitutions in the S gene region with an allele frequency $>$ 5 %. The mutation proportion was calculated as the number of variant reads relative to the number of reads at that position.

Hepatitis B virus quasispecies characteristics were evaluated at two levels: Complexity and diversity. Quasispecies complexity, also known as Shannon entropy, quantifies the distribution of mutant genomes within a population. It is calculated using the formula: $Sn = -[\sum i\ (pi \times lnpi)]/lnN$, where N is the total number of clones and pi is the frequency of each clone within the viral quasispecies population. (Chen et al., 2009) Quasispecies diversity is the association among individuals within the population, including the mean genetic distance (d), synonymous substitutions per synonymous site (dS), and nonsynonymous substitutions per nonsynonymous site (dN). Genetic distances were calculated using the Tamura 3-parameter model, incorporating transitional and transversional rates and $G+C$ content bias. The dS and dN were calculated using the modified Nei–Gojobori model with Jukes–Cantor correction in MEGA software (Tamura et al., 2013)

2.5. Statistical analyses

The χ^2 test or Fisher exact test was used to analyze categorical variables to compare group characteristics. Qualitative variables are reported as absolute (n) and relative (%) frequencies, while quantitative variables are reported as the mean, standard deviation, and median. The paired *t*-test or analysis of variance was used to compare variable values. Differences were considered statistically significant at *P*-values of $<$ 0.05.

2.6. Ethics statement

Informed consent was obtained from all participants or their first-degree relatives if the medical condition of the patient impaired their ability to provide consent. This study was approved by the research ethics committee of the Fourth Military Medical University, and all participants signed informed consent forms before participation.

3. Results

3.1. Occurrence of HBsAg and anti-HBs coexistence in patients in the acute phase

Among 215 adults diagnosed with acute phase HBV, 19 (8.84 %) cases demonstrated HBsAg and anti-HBs coexistence. Patients with HBsAg and anti-HBs coexistence exhibited a lower proportion of positive HBeAg compared with those with positive HBsAg and negative anti-HBs (92/196 versus 3/19, $\chi^2 = 6.185$, $P = 0.014$).

3.2. Occurrence of HBsAg and anti-HBs coexistence in patients in the chronic phase

During a median follow-up of 14 months (interquartile range: 14–28), 54 new cases of HBsAg and anti-HBs coexistence were identified among 4593 HBV carriers. The average annual incidence of coexistence was 1.41 % ± 0.28 %. In a Kaplan-Meier survival curve, the incidence

rate was linear over the course of the follow-up, and the cumulative incidence rate after 13 month, 27 month and 40 month was 1.65 % (95 % CI: 0.74–2.55), 3.96 (95 % CI:2.20–5.72), 4.59 (95 % CI:2.37–6.81) (Fig. 1). Dynamic observation of clinical characteristics revealed no significant difference in HBsAg and ALT levels after anti-HBs became positive. However, the HBV DNA level was significantly decreased ($t = 3.26$, $P < 0.01$) (Table 1). Among patients with HBeAg positivity, there was a non-significant difference in the HBeAg-negative conversion rate between those with anti-HBs positivity and those without (6/18 versus 209/598, $P = 0.89$).

3.3. Risk factors for the coexistence of HBsAg and anti-HBs in the chronic phase

Table 2 presents the risk factors for the coexistence of HBsAg and anti-HBs. Anti-HBs production was associated with ages of ≥ 50 years (risk ratio [RR]: 1.79, 95 % CI: 1.04–3.09, $P = 0.035$), HBeAg positivity (RR: 3.43, 95 % CI: 1.91–6.19, $P < 0.001$), baseline ALT abnormalities (RR: 3.62, 95 % CI: 1.93–6.79, $P < 0.001$), and higher HBV DNA level (RR: 1.97, 95 % CI: 1.12–3.49, $P = 0.017$).

3.4. Dynamic changes in mutations in the "a" determinant during anti-HBs conversion

Six patients who became anti-HBs positive during follow-up were selected. Table 3 summarizes their demographic and clinical data. All six patients demonstrated mutations in the "a" determinant, with 4/6 exhibiting wild-type predominance and 2/6 exhibiting "a" determinant mutant predominance (Fig. 2). The viral population displayed no significant changes after anti-HBs became positive. Additionally, non-significant changes were observed in quasispecies complexity (at aa level: $P = 0.29$, at nt level: $P = 0.42$) or quasispecies diversity (genetic distance at aa level: $P = 0.65$, genetic distance at nt level: $P = 0.79$, dS: $P = 0.98$, dN: $P = 0.65$) (Table 4).

3.5. Long-term outcomes of carriers with HBsAg and anti-HBs coexistence

We recruited 122 carriers with HBsAg and anti-HBs coexistence during the first serosurvey[8]. Fifty-eight patients were followed up for 2.9 ± 1.49 years. Among them, 15 (18.05 %) demonstrated negative anti-HBs, 4 (7.69 %) negative HBsAg, and 39 (74.26 %) maintained HBsAg and anti-HBs coexistence. During follow-up, patients with persistent HBsAg and anti-HBs coexistence exhibited non-significant

Table 1
Change in the clinical characteristics of new patients with HBsAg and anti-HBs of coexistence during anti-HBs development ($n = 54$).

	Before anti-HBs development	After anti-HBs development	t	*P*
HBsAg level (log IU/mL)	3.36 ± 0.51	3.16 ± 0.74	1.61	0.12
anti-HBs level (log IU/L)	0.47 ± 0.26	1.34 ± 0.34	12.27	< 0.01
HBV DNA level (log IU/mL)	4.66 ± 2.25	3.85 ± 2.31	3.26	< 0.01
ALT level (IU/L)	53.87 ± 98.78	43.39 ± 30.00	0.63	0.54

Table 2
Risk factors for anti-HBs development in HBsAg positive carriers becoming positive for both HBsAg and anti-HBs.

	Number of participants ($n = 4539$)	Number of the anti-HBs Appearance ($n = 54$)	Rate ratio of anti-HBs appearance	*P*
Gender				
Male	2237	23	1.00	
Female	2312	31	1.30 (0.76–2.24)	0.336
Age				
< 50	3278	32	1.00	
≥ 50	1261	22	1.79 (1.04–3.09)	0.035
HBeAg				
Negative	3715	34	1.00	
Positive	541	17	3.43 (1.91–6.19)	< 0.001
Baseline ALT levels, IU/L *				
Normal	2272	13	1.00	
Abnormal	1932	40	3.62 (1.93–6.79)	< 0.001
Baseline HBV DNA levels, IU/mL*				
< 20,000	2169	31	1.00	
≥ 20,000	709	20	1.97 (1.12–3.49)	0.017

* The data of HBeAg, ALT, and HBV DNA in some cases were missed because of insufficient blood samples.

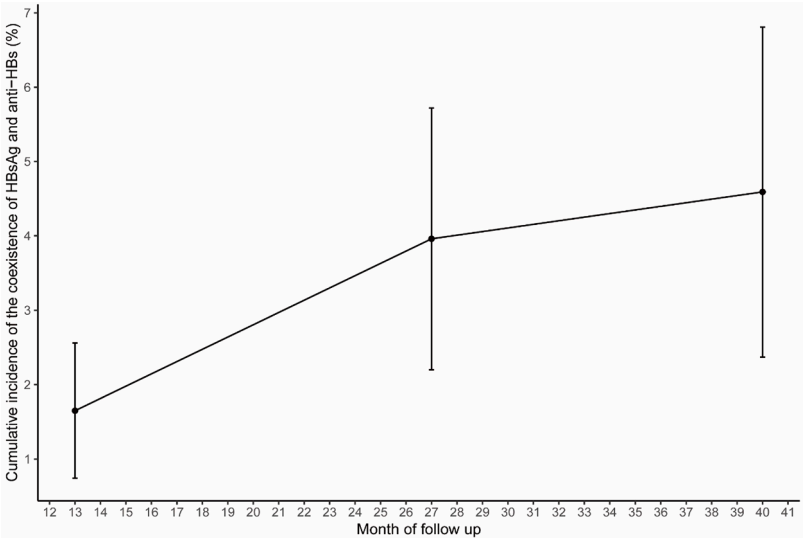

Fig. 1. Cumulative incidence rates of anti-HBs development in patients with positive HBsAg becoming positive for both HBsAg and anti-HBs ($n = 4593$).

Table 3
Demographic, virological, and clinical characteristics of six patients with anti-HBs seroconversion.

		Patient 1	Patient 2	Patient 3	Patient 4	Patient 5	Patient 6
Age, year		60	52	41	40	47	37
Gender		Male	Male	Female	Female	Female	Female
HBsAg	13 months	4448.00	> 5000	> 5000	444.50	3127.00	3035.00
IU/mL	27 months	2663.00	4286.00	> 5000.00	677.10	> 5000.00	> 5000.00
	40 months	4212.00	> 5000.00	4785.00	906.30	> 5000.00	/
Anti-HBs	13 months	< 2.00	40.34	< 2.00	4.07	< 2.00	5.44
IU/L	27 months	< 2.00	8.86	7.03	12.20	25.27	135.60
	40 months	11.97	25.33	21.22	40.83	28.57	/
HBeAg	13 months	Negative	Negative	Positive	Positive	Negative	Positive
	27 months	Negative	Negative	Positive	Positive	Negative	Negative
	40 months	Negative	Negative	Negative	Positive	Negative	/
HBV DNA	13 months	3.62	Negative	4.05	7.78	5.83	3.60
log IU/mL	27 months	3.02	Negative	3.39	7.93	6.26	2.89
	40 months	4.36	Negative	3.19	8.47	5.88	/
ALT	13 months	25	37	7	29	37	178
IU/L	27 months	40	37	27	50	27	94
	40 months	34	30	23	36	42	/

"/" means that the data are missing because of fewer blood samples.

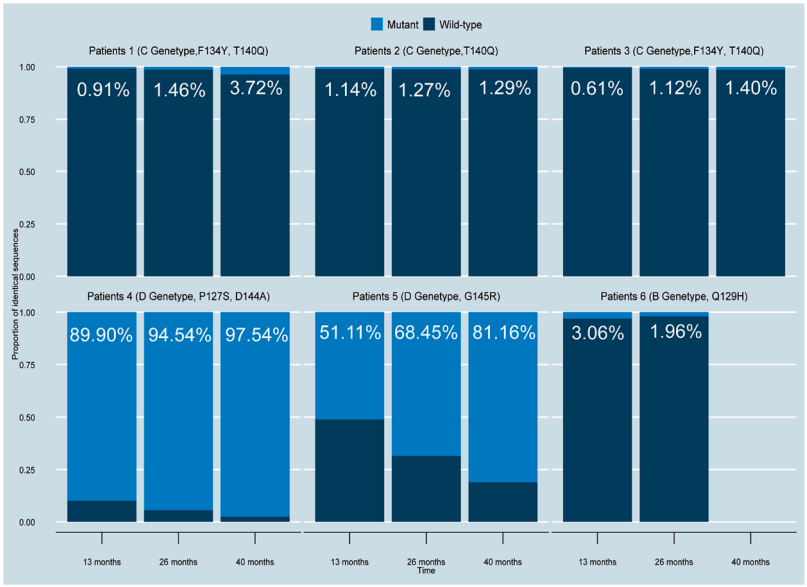

Fig. 2. Dynamics of the proportion of "a" determinant mutations following anti-HBs seroconversion in HBsAg carriers. Identical sequences represent amino acid sequences matching the reference sequences. (Reference B: AB073846, Reference C: AB014381, Reference D: X85254).

Table 4
Quasispecies complexity and diversity of the MHR region of S gene in HBsAg carriers after anti-HBs development ($n = 6$).

	Anti-HBs negative	Anti-HBs positive	*P*
Quasispecies complexity (aa level)	0.75 ± 0.21	0.88 ± 0.08	0.29
Quasispecies complexity (nt level)	0.69 ± 0.29	0.84 ± 0.16	0.42
Genetic distance (10^{-3} substitution/ sites, aa level)	6.50 ± 8.60	7.00 ± 10.94	0.65
Genetic distance (10^{-3} substitution/ sites, aa level)	3.20 ± 4.00	3.10 ± 4.60	0.79
dS (10^{-3} substitution/site)	3.45 ± 4.71	3.70 ± 4.35	0.98
dN (10^{-3} substitution/site)	3.10 ± 3.93	3.30 ± 4.98	0.65

aa: Amino acid; nt: Nucleotide.

changes in HBsAg levels (3.10 ± 0.73 versus 3.27 ± 0.56 versus 3.03 ± 0.80 log IU/mL, $F = 0.78$, $P = 0.46$), anti-HBs level (1.71 ± 0.51 versus 1.47 ± 0.57 versus 1.57 ± 0.60 log IU/L, $F = 1.11$, $P = 0.34$), HBV DNA level (3.92 ± 2.06 versus 4.00 ± 2.43 versus 3.40 ± 2.69 log IU/mL, $F = 0.42$, $P = 0.66$), and ALT level (34.00 ± 28.86 versus 38.64 ± 38.97 versus 40.21 ± 22.07 IU/L, $F = 0.39$, $P = 0.68$).

4. Discussion

HBsAg and anti-HBs coexistence is an unusual serological pattern in HBV infection, and its natural course remains poorly understood. This study investigated the occurrence of this pattern in patients with HBV in acute and chronic phases and the kinetics changes of HBV serological markers during the chronic phase.

In this study, the natural incidence of HBsAg and anti-HBs coexistence in HBV infection was first reported. Follow-up with HBV carriers revealed an annual incidence of 1.41 % for this coexistence during the chronic phase. Among adult patients in the acute phase of HBV infection, the coexistence rate was 8.84 %, significantly higher than the prevalence observed in the chronic phase in previous studies (2.4 %–5.8 %) (Lee et al., 2020; Wang et al., 2022; Hou et al., 2020; Jiang et al., 2021). These findings suggest that HBsAg and anti-HBs coexistence may represent an intermediate stage in the natural history of HBV. The new HBsAg assay format and sensitivity improvements enable HBsAg detection in immune complexes. Qualitative assays for HBsAg had the

ability to detect classical mutations in the MHR (Lou et al., 2011), quantitative assays for HBsAg from coexistence patients were performed favorable and the circulating anti-HBs did not influence HBsAg quantification (Pancher et al., 2015). The improvement of diagnosis methods rather than faulty methodology in HBsAg or anti-HBs detection could primarily account for the coexistence.

The present study identified an association between ages ≥ 50 years and the occurrence of HBsAg and anti-HBs coexistence. Older patients demonstrated a higher prevalence of this coexistence compared with younger individuals, consistent with findings from a previous study (Pu et al., 2016). Older age has been recognized as a significant factor associated with HBsAg clearance (Simonetti et al., 2010; Ahn et al., 2005). Studies involving cohorts with a mean age of 40 years or older reported higher rates of HBsAg clearance than those with younger cohorts, with annual rates of 1.7 % in cohorts aged ≥ 40 years compared with 1.1 % in younger cohorts (Song et al., 2021). The age-dependent disparity in the clinical course of chronic HBV infection, characterized by replicative and nonreplicative phases, is attributable to the disease phase of the HBV infection. (EASL 2012)

Our study also revealed that patients with positive HBeAg, baseline ALT abnormalities, and higher HBV DNA levels demonstrated a higher rate of anti-HBs development. Traditionally, the phases of chronic HBV infection are categorized based on HBeAg status, serum HBV DNA levels, and ALT levels into five phases. Patients transition from a state of high viral load with no liver disease to active liver disease, followed by an inactive disease phase, and then revert to active liver disease years later. The HBeAg-positive chronic hepatitis phase is characterized by HBeAg presence, high HBV DNA levels, and elevated ALT (EASL 2017). Our data indicated that the coexistence of HBsAg and anti-HBs may mainly occur during the HBeAg-positive chronic hepatitis phase.

Additionally, our study observed no significant changes in the quasispecies heterogeneity of mutations in the “a” determinant during the occurrence of coexistence with HBsAg and anti-HBs in HBV infection. Previous studies identified the “a” determinant mutations as important in HBsAg and anti-HBs coexistence (Gu et al., 2024). The frequency of viral quasispecies within the “a” determinant significantly changes under antiviral treatment or immune pressure (do Lago et al., 2023). G145R mutation which observed in patient 5 in our study was widely reported as the most common immune-escape mutation. It could dramatically decreases the affinity for anti-HBs and leads to the diagnostic failure of commercial assays for HBsAg. Although the proportion of G145R in patient 5 changed from 51.11 % to 81.16 % during the anti-HBs conversion in our study, the HBV DNA load maintained at high level and there was virus replication. This demonstrated that G145R are more likely to be associated with the diagnostic failure but not the coexistence of HBsAg and anti-HBs. Our analysis revealed that immune escape mutant selection was not achieved during anti-HBs production. A recent study indicated that patients with hepatocellular carcinoma exhibit higher rates of HBsAg mutations (Thi Cam Huong et al., 2022). Further investigation is needed to clarify the association between “a" determinant mutations and HBsAg and anti-HBs coexistence while accounting for confounding factors that elevate mutation rates.

In this study, 7.69 % of carriers with HBsAg and anti-HBs coexistence achieved HBsAg seroclearance. Previous studied reported that the coexistence of HBsAg and anti-HBs increased the risk of HCC development (Jin et al., 2019; Seo et al., 2014). This indicated that the coexistence of HBsAg and anti-HBs was associated not only with HBsAg seroclearance but also with HCC. Our study demonstrated that the HBV DNA remained detectable in patients with HBsAg and anti-HBs coexistence and exhibited active HBV replication. This underscores the heterogeneous nature of HBsAg and anti-HBs coexistence. While some patients with this coexistence progress to HBsAg clearance, others may experience adverse outcomes due to positive HBeAg and high HBV DNA levels. Additionally, Nobukazu et al. reported that patients with acute HBV infection may remain HBV DNA-positive for different periods despite previously developing anti-HBs (Yuki et al., 2003).

This study has some limitations that should be addressed. First, longitudinal outcomes, including the incidence of HCC, were not evaluated in patients with HBsAg and anti-HBs coexistence. Second, the immune profile reflecting host immune responses was not evaluated in all participants. Third, longitudinal analysis of a “determinant” sequence diversity was evaluated in a very small number of patients. Future studies should aim to elucidate the immune characteristics associated with the occurrence of HBsAg and anti-HBs coexistence.

In conclusion, HBsAg and anti-HBs coexistence can occur in both acute and chronic phases of HBV infection. Its occurrence is associated with patient age, HBeAg status, HBV DNA levels, and ALT levels. The viral quasispecies of “a” determinant mutation demonstrated no changes during the occurrence of coexistence with HBsAg and anti-HBs in HBV infection. These findings indicate that HBsAg and anti-HBs coexistence may represent a natural course of the HBV infection, corresponding to its disease phase.

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Data availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

CRediT authorship contribution statement

Zhongshu Pu: Writing – original draft, Data curation. **Zhaohua Ji:** Data curation. **Haixia Su:** Methodology, Formal analysis. **Ting Fu:** Software, Data curation. **Zhongjun Shao:** Writing – review & editing. **Yongping Yan:** Writing – review & editing, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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