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Evaluation of reverse transcription-polymerase chain reaction and simultaneous amplification and testing for quantitative detection of serum hepatitis B virus RNA

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A R T I C L E I N F O

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A B S T R A C T

Background: Chronic hepatitis B virus (HBV) infection is one of the common infectious diseases in the world. HBV covalently closed circular DNA (cccDNA) is the initial template of HBV replication, which can exist in human hepatocytes for a long time and is difficult to be completely removed. It has been shown that HBV RNA can directly respond to the levels and transcriptional activity of cccDNA in hepatocytes and can be used as a surrogate marker of cccDNA transcriptional activity. At present, the detection techniques used for quantitative HBV RNA mainly include reverse transcription quantitative polymerase chain reaction (RT-qPCR) and simultaneous amplification and testing (SAT).

Methods: In this study, we verified the performance of the SAT method for detecting HBV RNA and the clinical effectiveness of SAT and RT-qPCR, and compared the correlation and consistency of the two detection methods for HBV RNA detection.

Results: The results showed that the limit of detection for HBV RNA by SAT method was 50 copies/mL, with a linear range of 1×10^2-1×10^8 copies/mL. There was no difference in HBV RNA levels detected by the two methods. The correlation and consistency of the results were good, with the coefficient of determination of 0.7787 in HBeAg positive group and 0.8235 in HBeAg negative group.

Conclusions: Therefore, this study confirmed that the SAT method and RT-qPCR for detecting HBV RNA have good agreement, which are both reliable methods to detect HBV RNA and can replace each other.

1. Introduction

Chronic hepatitis B (CHB) and its related diseases are currently major public health problems in the world [1,2]. It is a

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well-recognized fact that hepatitis B virus (HBV) infection is difficult to completely cure, due to the persistence of covalently closed circular DNA (cccDNA) in the nucleus of infected hepatocytes [2–4]. At present, the main therapeutic drugs for CHB are nucleotide analogues (NAs) and PEG based interferon (Peg IFN) [5]. The goal of antiviral therapy for hepatitis B is to maximize the long-term inhibition of HBV replication. The complete elimination of cccDNA in liver tissue is an important sign of the cure of HBV infection [6]. Currently, liver cccDNA is mainly detected by liver puncture, but it is difficult to be widely used in clinical practice because of its invasiveness [7,8]. Therefore, accurate evaluation of the expression level and transcriptional activity of cccDNA in the liver is an urgent clinical problem.

In HBV infection and replication, cccDNA acts as a transcription template to transcribe more than five kinds of mRNAs with different lengths, of which 3.5 kb pregenomic RNA (pgRNA) can not only translate and synthesize HBV core antigen (HBcAg) and P proteins, but also act as a viral reverse transcription template to synthesize viral negative strand DNA [9,10]. Studies have confirmed that HBV RNA in the serum of HBV infected persons was derived from HBV pgRNA [11]. HBV RNA, which reflects the activity of cccDNA in the liver, is also highly associated with traditional markers such as HBV surface antigen (HBsAg) and HBV DNA [12,13]. It can also predict the HBV e antigen (HBeAg) seroconversion during antiviral therapy and the virological relapse and HBsAg reversal after drug withdrawal [14,15]. Serum HBV RNA has been included in the Chinese and European guidelines for hepatitis B prevention and treatment as an alternative serological indicator of cccDNA [16,17]. Therefore, HBV RNA is expected to become an important marker for achieving accurate treatment of CHB.

Currently, there is no standardized HBV RNA detection method in the world. The HBV RNA detection methods mainly include rapid amplification of eDNA ends (RACE), reverse transcription quantitative polymerase chain reaction (RT-qPCR), simultaneous amplification testing (SAT), reverse transcriptase droplet digital polymerase chain reaction (RT-ddPCR) and the QuantiGene determination technique [12,18]. In this study, we evaluated the performance of the SAT method detection kit, and compared the consistency evaluation and substitutability of HBV RNA detected by SAT and RT-qPCR in clinical application.

2. Materials and methods

2.1. Research objects

In this study, 88 patients with CHB without antiviral treatment hospitalized in Nanjing Drum Tower Hospital (Nanjing, China) from April 2017 to December 2020 were selected. The diagnosis criteria of CHB were judged according to the Guidelines for the Prevention and Treatment of Chronic Hepatitis B (2015). Inclusion criteria: (1) Patients met the diagnostic criteria of CHB; (2) Patients were positive for HBsAg for more than 6 months. Exclusion criteria: (1) Patients infected with other viruses, including hepatitis C virus, hepatitis D virus and human immunodeficiency virus; (2) Patients with decompensated cirrhosis, liver cancer and other malignant tumors; (3) Patients with other chronic liver diseases, including primary biliary cirrhosis, autoimmune hepatitis, alcoholic liver disease, and nonalcoholic fatty liver; (4) Patients who received liver transplantation before enrollment. The study was approved by the Ethics Committee of Nanjing Drum Tower Hospital, and all included subjects have signed informed consent form (Ethical number: 2008022).

2.2. Specimen collection

For each enrolled patient, 5 mL blood sample were collected with a vacuum blood collection tube. After resting at room temperature for 30 min, the serum was collected by centrifugation at 3000 rpm for 15 min and stored at −80 °C. The serum was divided into 2 aliquots, and the HBV RNA in serum was determined by RT-qPCR and SAT respectively.

2.3. Reagents and instruments

The instrument used for RT-qPCR is Applied Biosystems 7500 (ABI 7500, USA). The reagents of nucleic acid extraction, HBV RNA and HBV DNA quantitative detection were purchased from Hunan Shengxiang Biotechnology Co., Ltd. The SAT method used the AutoSAT full-automatic nucleic acid analysis system of Shanghai Rendu Biotechnology Co., Ltd. The HBV RNA detection reagent was purchased from Shanghai Rendu Biotechnology Co., Ltd. HBV RNA standards were obtained from the respective kits. Serum HBsAg and HBeAg were determined using the Architect i2000SR platform and Abbott Architect reagents (Abbott Laboratories, Chicago, IL).

2.4. RT-qPCR

Nucleic acids from the samples were extracted according to the reagent instructions, including patient samples, HBV RNA quantitative references A-D, negative control and positive control. 20 μL of nucleic acid was added to the configured 30 μL PCR reaction solution for testing. The results were analyzed according to the reagent instructions and instrument instructions.

2.5. SAT

A total 400 μL of serum was put into the AutoSAT full-automatic nucleic acid detection and analysis system for detection according to the standard operation process of the instrument and the reagent instruction manual. The extraction, amplification and detection of nucleic acid were automatically completed in the instrument. The quantitative results were calculated by the instrument software and

displayed in the result interface.

2.6. The sensitivity and linear range of RT qPCR and SAT methods

The sensitivity and linear range of RT qPCR and SAT methods were shown in Table 1. For the samples with the lower limit of detection (LLOD) and lowest linear range, the detection were repeated three times and took the average value. The samples exceeding the detection limit were diluted with negative serum and tested in triplicate.

2.7. Statistical analysis

Before statistical analysis, the data of serum HBV RNA and HBV DNA were logarithmically transformed. The characteristics of the participants were expressed in proportion, mean or median (IQR). The HBV RNA and HBV DNA results as well as the correlation of the two HBV RNA detection results were analyzed by linear regression. The consistency of the two detection methods in HBV patients was expressed by Bland Altman diagram. $P < 0.05$ means the difference is statistically significant. Statistical analysis was conducted on Microsoft Office Excel, Graphpad Prism 9 and R4.1.3.

3. Results

3.1. Clinical characteristics of CHB patients and control group

A total of 110 samples were enrolled in this study, including 88 CHB patients and 22 health examiners, and the data of all the enrolled samples were shown in Table 2.

In this study, 61 of CHB patients were HBeAg positive, and 27 patients were HBeAg negative. The levels of HBV DNA and HBsAg in HBeAg positive patients were 7.78 [7.39; 8.03] $\log_{10}$ copies/mL and 4.62 [4.30; 4.81] $\log_{10}$IU/mL, respectively, which were significantly higher than those in HBeAg negative patients (5.18 [3.57; 5.97] $\log_{10}$ copies/mL and 3.29 [3.05; 3.72] $\log_{10}$ IU/mL, respectively, $P < 0.001$). Moreover, the levels of HBV RNA in HBeAg positive group detected by SAT and RT-qPCR were significantly higher than those in HBeAg negative group ($P < 0.001$). Specifically, the HBV RNA level in HBeAg positive group was 7.17 [6.72; 7.43] $\log_{10}$ copies/mL or 7.17 [6.72; 7.43] $\log_{10}$ copies/mL, while it was 4.74 [3.95; 5.42] $\log_{10}$ copies/mL or 4.22 [3.30; 5.80] $\log_{10}$ copies/mL in HBeAg negative group. Characterization of HBeAg positive and HBeAg negative CHB patients were listed in Table 3.

3.2. The linear range of SAT for detecting HBV RNA

To validate the linear range of SAT, we diluted the HBV RNA standard (1×10^8 copies/mL) gradient into different concentrations with negative serum, including 1×10^7, 1×10^6, 1×10^5, 1×10^4, 1×10^3 and 1×10^2 copies/mL. Each concentration was tested three times according to the instructions, and the log deviation value of the actual measured concentrations and the theoretical concentrations of each dilution was required to be within the range of ± 0.5. As shown in Fig. 1, the correlation coefficient of the log value of the theoretical concentration and the actual concentration was 0.9833, and the linear range of SAT method for detecting HBV RNA was 1×10^2-1×10^8 copies/mL, consistent with the linear range stated in reagent instruction.

3.3. The sensitivity and specificity of SAT for detecting HBV RNA

The sensitivity test for SAT assay was to dilute the HBV RNA standard to 50 copies/mL with negative serum and repeated the detection 20 times. All the 20 results were positive, indicating that the limit of detection was 50 copies/mL. Furthermore, we tested the serum HBV RNA in 22 healthy people and none of them were detected, confirming the specificity of this assay.

3.4. The accuracy and repeatability of SAT for detecting HBV RNA

The HBV RNA standard was diluted into three gradient concentrations of 1×10^5, 1×10^4 and 1×10^3 copies/mL for the accuracy study. The results showed that the absolute deviation of the log value of measured concentration and theoretical concentration was within ± 0.5, meeting the performance requirements of the accuracy (Table 4).

The repetitive performance assessment was performed by diluting the HBV RNA standard to 1×10^3 copies/mL with negative serum and repeated the testing 10 times. The coefficient of variation (CV) of the log value of measured concentrations was 4%, indicating the good repeatability of the method (Table 5).

Table 1
Sensitivity and linear range of RT-qPCR and SAT.

Methods	Sample volume (μL)	Limit of detection (copies/mL)	Linear range (copies/mL)
RT-qPCR	20	50	1×10^2–1×10^8
SAT	400	50	1×10^2–1×10^8

Table 2
Characterization of CHB patients and control group.

Variables	CHB patients	Control group
	N = 88	N = 22
Gender		
Female	42 (47.7%)	12 (54.5%)
Male	46 (52.3%)	10 (45.5%)
Age (year)	35.0 [31.0; 55.2]	57 [39.0; 6 2.7]
AST (U/L)	31.6 [22.5; 48.1]	10.3 [4.0; 18.9]
ALT (U/L)	43.0 [24.9; 68.2]	12.5 [5.6; 22.1]
HBV DNA (log_{10} copies/mL)	7.44 [5.93; 7.93]	not detected
HBsAg (log_{10} IU/mL)	4.33 [3.54; 4.72]	not detected
HBeAg (S/CO)	3.05 [0.00; 3.16]	not detected

HBV: hepatitis B virus; AST: aspartate aminotransferase; ALT: alanine aminotransferase; HBsAg: hepatitis B surface antigen; HBeAg: hepatitis B e antigen. All expressed in median. The data expressed as Median (IQR).

Table 3
Characterization of HBeAg positive and HBeAg negative CHB patients.

Variables	HBeAg negative	HBeAg positive	p.overall
	N = 27	N = 61	
Gender			0.042
Female	8 (29.6%)	34 (55.7%)	
Male	19 (70.4%)	27 (44.3%)	
Age (year)	56.0 [40.0; 63.0]	32.0 [29.0; 38.0]	<0.001
AST (U/L)	35.7 [29.0; 55.9]	28.0 [21.6; 44.3]	0.141
ALT (U/L)	49.0 [35.1; 101]	36.8 [24.6; 63.7]	0.153
HBV DNA (log_{10} copies/mL)	5.18 [3.57; 5.97]	7.78 [7.39; 8.03]	<0.001
HBV RNA (log_{10} copies/mL) by SAT	4.74 [3.95; 5.42]	7.17 [6.72; 7.43]	<0.001
Detectable	27/27	61/61	
HBV RNA (log_{10} copies/mL) by RT-qPCR	4.22 [3.30; 5.80]	7.22 [6.90; 7.47]	<0.001
Detectable	27/27	61/61	
HBsAg (log_{10} IU/mL)	3.29 [3.05; 3.72]	4.62 [4.30; 4.81]	<0.001
HBeAg (S/CO)	0	3.14 [3.03; 3.18]	NA

NA：Not applicable; the data expressed as Median (IQR).

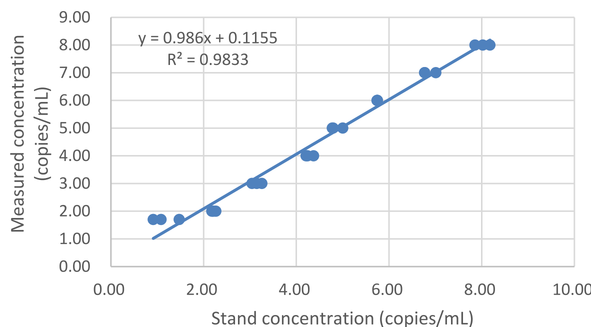

Fig. 1. Linear range of SAT for detecting HBV RNA.

Table 4
Accuracy detection of the SAT for detecting HBV RNA.

Stand concentration (copies/mL)	Measured concentration (copies/mL)					Mean (copies/mL)	SD
	Repeat 1	Repeat 2	Repeat 3	Repeat 4	Repeat 5		
3.00	3.05	3.26	3.15	3.44	3.36	3.22	0.16
4.00	4.21	4.24	4.37	4.30	3.91	4.28	0.18
5.00	5.00	4.78	4.80	5.15	4.94	4.93	0.15

3.5. Correlation and consistency of HBV RNA detected by RT-qPCR and SAT

The linear correlation analysis of HBV RNA detected by RT-qPCR and SAT showed a significant correlation ($R^2 = 0.9037$). The linear regression equation was Y = 1.012*X-0.0988 (Fig. 2). The consistency of the two methods in CHB patients was represented by Bland Altman diagram (Fig. 3). The scatter plot took the difference between the two measurement results as the vertical axis and the mean of the measurement results as the horizontal axis and the 95% limit of agreement (95% LoA) were marked with dotted lines. From the results, the range of 95% LoA was narrow, with a difference of −0.9512 to 1.004 log_{10} copies/mL. Based on RT-qPCR method, the deviation value of SAT method in CHB patients was 0.026, so it can be considered that the consistency of the two detection values is good.

3.6. Correlation and consistency of HBV RNA detected by RT-qPCR and SAT in HBeAg positive and HBeAg negative groups

Compared with the HBeAg negative group, the HBeAg positive group had higher HBV RNA level (7.17 log_{10} copies/mL vs 4.34 log_{10} copies/mL), which was similar to the distribution of HBV DNA. There was no statistically significant difference in HBV RNA level detected by the two methods (Fig. 4A and D). In HBeAg negative group, SAT method had more positive results and higher median. The median of the SAT method was 4.34 log_{10} copies/mL, and the RT-qPCR method was 3.79 log_{10} copies/mL ($p = 0.80$). In HBeAg positive group, the median of SAT was 7.17 log_{10} copies/mL, and the RT-qPCR was 7.22 log_{10} copies/mL ($P = 0.425$). We also analyzed the linear correlation of HBV RNA detected by RT-qPCR and SAT in the HBeAg positive and HBeAg negative groups, showing high correlations ($R^2 = 0.7787$ and $R^2 = 0.8235$, respectively) (Fig. 4B and E). Moreover, as shown in Fig. 4C and F, the deviation of both methods was less than 2. Therefore, SAT and RT-qPCR had good consistency when detecting patients with HBeAg positive and negative. Howerer, there was a certain deviation in the two methods, with 6.56% (4/61) and 3.03% (1/33) of the points not within the 95% LoA, respectively.

4. Discussion

At present, the clinical use of NAs to treat CHB still takes HBV DNA inhibition as the end point of treatment, but studies found that the failure of HBV DNA detection does not indicate cccDNA clearance or inhibition of cccDNA transcriptional activity [19]. In addition, some studies have shown that there is a good correlation between the level of HBsAg and cccDNA, but some HBsAgs in the plasma of CHB patients may come from the integrated HBV DNA, so HBsAg is not reliable as a surrogate indicator of cccDNA [20,21]. A large number of clinical studies have confirmed that HBV RNA can reflect the transcriptional activity of cccDNA, which makes HBV RNA to become a more reliable substitute for cccDNA [22,23].

At present, the HBV RNA detection methods mainly include RACE, RT-qPCR, SAT, RT-ddPCR and QuantiGene [24]. Some studies found that there are a large number of HBV RNA without poly a tail in serum, which may lead to missed detection using RACE technology [25]. RT-ddPCR can improve the sensitivity and specificity of serum HBV RNA detection, which is superior to RT-qPCR [26,27]. SAT has simple requirements for instruments and operations, with the advantages of high sensitivity, high specificity and low pollution in rapidly detection [28]. Due to the lack of standardization, the clinical results of HBV RNA are heterogeneous, and the impact of different detection methods on the differences in research results still needs to be explored.

In this study, the detection performance of SAT was evaluated, and 88 CHB patients with HBsAg positive ($n = 61$) and HBsAg negative ($n = 27$) were detected by RT-qPCR and SAT, respectively. By analyzing the results of two parallel tests of 88 serum samples from CHB patients, we found a good correlation between SAT method and traditional RT-qPCR method. It showed that the two methods have good comparability and can accurately reflect the serum HBV RNA level in vivo. Further, the correlation of the two methods in HBeAg positive group was better than that of HBeAg negative group, probably due to the higher cccDNA levels and transcriptional activity in the liver of HBeAg positive patients [13]. Recent studies have determined that HBeAg negative patients have significantly lower cccDNA transcriptional activity, lower overall pgRNA activity, and some patients have one or more steps to inhibit the HBV DNA replication downstream of pgRNA, so that HBV RNA may lack some functional structures [29,30]. The HBV RNA in

Table 5
Repeatability detection of the SAT for detecting HBV RNA.

Measured concentration (copies/mL)	Log-transformed measured concentration (log_{10} copies/mL)
2.75E+03	3.44
2.30E+03	3.36
2.18E+03	3.34
2.37E+03	3.37
2.71E+03	3.43
1.79E+03	3.25
2.64E+03	3.42
1.56E+03	3.19
1.15E+03	3.06
1.40E+03	3.15
X	3.30
SD	0.13
CV	0.04

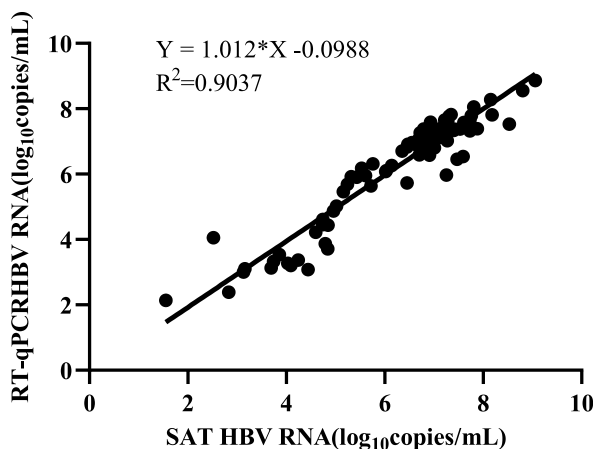

Fig. 2. Correlation of SAT and RT-qPCR in 88 CHB patients.

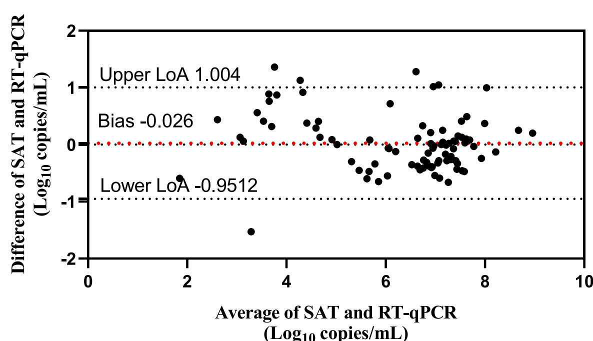

Fig. 3. Consistency of SAT and RT-qPCR in 88 CHB patients.

peripheral blood is mainly pgRNA. However, in addition to the 3.5 kb pgRNA and its reverse transcriptional residues, there are also different forms of pgRNA splicing variants and 3 ′end truncated bodies in the peripheral blood. Therefore, to be exact, HBV RNA is actually a mixture [24]. The detected HBV RNA will vary with different reagent brands and different detection methods using primers and/or probes at different positions.

For some individual samples, both methods failed to detect the HBV RNA, especially the RT-qPCR, which may be due to the inevitable loss of RNA during DNA digestion, reducing the detection sensitivity. And the residual DNA and HBV RNA can be amplified together, affecting the accuracy of quantification. However, the AutoSAT platform is a fully automatic detection system. Reverse transcription and amplification can be carried out simultaneously in real time, with higher amplification efficiency. And the amplification product is RNA with fast degradation, reducing the risk of laboratory pollution.

We also found that the two methods can obtain more positive patients than HBV DAN in HBeAg positive and negative groups. Among the 9 HBV DNA negative patients, only 2 were negative by SAT method and 5 were negative by RT-qPCR. This may be due to the fact that HBV RNA is the downstream product of cccDNA, cccDNA depletion is also needed in HBeAg negative patients from HBV DNA negative to HBV RNA negative resulting a time difference.

The HBsAg level in HBeAg negative group was low. It was proposed that the low level of serum HBsAg (logarithm ≤3 IU/mL) could predict the future HBsAg seroconversion. Serum HBV RNA was significantly higher in patients with low HBsAg level (≤3 log IU/mL), making HBV RNA a potential clinical treatment and monitoring marker proved in HBeAg negative patients. It is important to find new biomarkers as indicators for initiating antiviral therapy, especially in this group of patients, as this situation may be related to more active liver virus replication, leading to the development of cirrhosis and hepatocellular carcinoma. In those patients without treatment indicators, high HBsAg levels or HBV RNA results may suggest that more frequent follow-up is needed. On the other hand, among, low HBsAg levels and no detection of HBV RNA also provided a basis for stopping treatment in HBeAg negative patients who stopped NAs treatment in accordance with the current guidelines. Studies have shown that the results of these markers may affect the treatment decisions in more than 50% (66/126) of HBeAg negative patients.

This study has certain limitations. We only compared two methods for HBV RNA detection and the sample size was not large enough. Next, more detection methods should be included, the sample detection scale should be expanded, and the performance of different detection systems should be comprehensively evaluated.

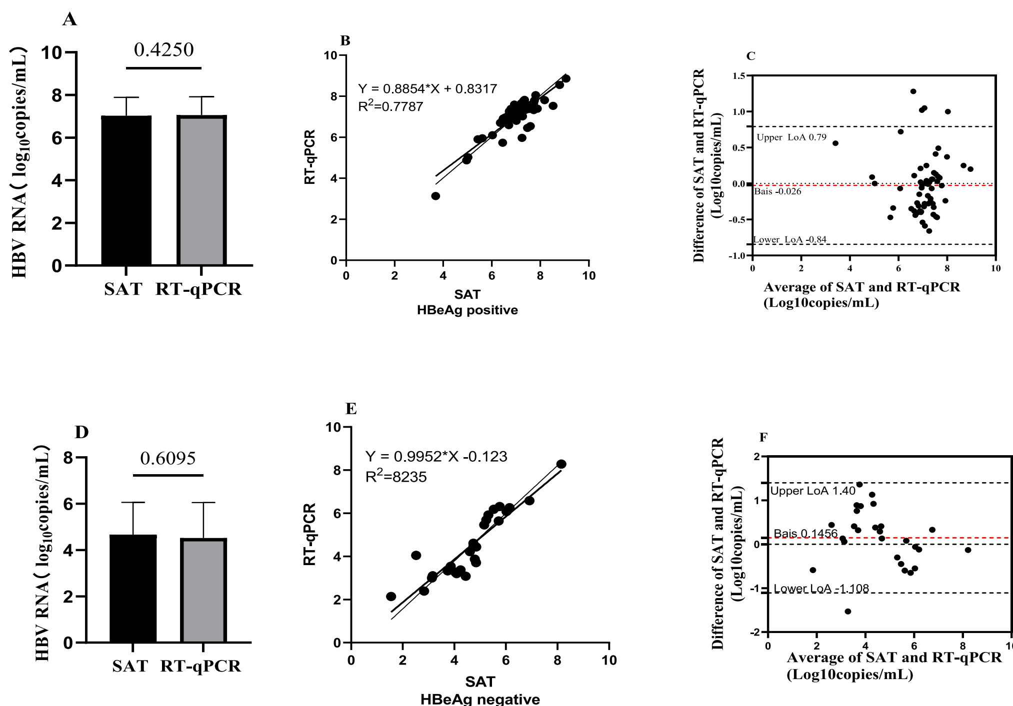

Fig. 4. Correlation and consistency of HBV RNA detected by SAT and RT-qPCR in HBeAg positive and HBeAg negative groups (a) HBV RNA detected by SAT and RT-qPCR in HBeAg positive group; (b) correlation analysis of the two methods in HBeAg positive group; (c) consistency analysis of the two methods in positive group; (d) HBV RNA detected by SAT and RT-qPCR in HBeAg negative group; (e) correlation analysis of the two methods in HBeAg negative group; (f) consistency analysis of the two methods in negative group.

5. Conclusion

To sum up, this study validated the performance of SAT for HBV RNA detection, compared the correlation and consistency between SAT method and RT-qPCR, and considered that the two methods are alternative. As a new marker, the methodology needs to be standardized to make the results generally recognized. It is hoped that HBV RNA will have international standard detection methods and standards like HBV DNA in the future.

Ethics statement

The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the Ethics Committee of Nanjing Drum Tower Hospital, and all included subjects have signed informed consent form (Ethical number: 2008022).

Author contribution statement

Xiaohan Hu; Miao Li: Analyzed and interpreted the data; Wrote the paper.
Liwei Zhao; Mingrong Ou: Performed the experiments.
Yuxin Chen; Hongxia Wei; Yanyan Xia: Contributed reagents, materials, analysis tools or data.
Hongpan Xu; Jun Wang: Conceived and designed the experiments.

Data availability statement

Data included in article/supp. material/referenced in article.

Additional information

No additional information is available for this paper.

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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