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Original Article/Liver

Exonuclease I and III improve the detection efficacy of hepatitis B virus covalently closed circular DNA

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ABSTRACT

Background: Hepatitis B virus covalently closed circular DNA (HBV cccDNA) is an important biomarker of hepatitis B virus infection. However, the current methods are not specific and sensitive. The present study aimed to develop a specific and sensitive assay method for the quantification of HBV cccDNA.
Methods: Exonuclease I (Exo I) & Exonuclease III (Exo III) and specific primer probes are used in real-time PCR. The virus particles isolated from peripheral blood mononuclear cells were used as negative control and HBV1.3 recombinant plasmid 3.2 kb circular DNA fragment was used as positive control. The methods of cccDNA detection were evaluated in cell lines, plasmid, animal model, patient serum and liver biopsies.
Results: A linear range of 10^1–10^7 copies/assay using specific primers for HBV cccDNA was established. HBV cccDNA were only detected in cell lines, animal model and liver tissue. It cannot be detected in serum samples. Intrahepatic HBV cccDNA level had good correlation with intrahepatic total HBV DNA level ($r = 0.765$, $P < 0.001$).
Conclusions: The real-time quantitative PCR is an effective and feasible method for sensitive and specific detection of low copy number of cccDNA. The novel detection method is fast, provides high sensitivity and specificity and can be used in clinical practice.

Introduction

Although prophylactic vaccine has been widely used, hepatitis B virus (HBV) infection remains a major concern of public health. It was estimated that 240 million people had chronic infections worldwide, and many of them eventually develop cirrhosis and even hepatocellular carcinoma (HCC) [1,2]. Although the approved therapeutics for chronic hepatitis B (CHB) is efficient enough to inhibit the replication of HBV and postpone the progression of liver diseases, they are unable to eradicate the virus. The patients may relapse after the termination of antiviral treatment. A key step in HBV life cycle is the formation of covalently closed circular DNA (cccDNA), which serves as the template for transcription of viral RNA and the reservoir of the virus. The replication of HBV is a unique process in the nucleus of hepatocytes. The presence of cccDNA can be interpreted as the lack of effectiveness of antiviral therapy for patients with chronic hepatitis B [3–5].

None of the current drugs eradicate cccDNA in infected hepatocytes [6]. Given the role of cccDNA in HBV life cycle, monitoring cccDNA levels help physician monitor the efficacy of antiviral treatment. However, this virological marker has not been widely used in clinical practice due to lack of feasible method of detection. Therefore, development of a reliable method for cccDNA detection is an unmet need in the research agenda for CHB infection [7–9].

Several methods have been developed to detect HBV cccDNA, including real-time polymerase chain reaction (PCR) and Southern blotting [10,11]. However, Southern blotting was not only inappropriate for quantification, but also too complicated to be used in clinical practice. As for real-time PCR, most of the existing methods were not satisfactory because of false positives caused by relaxed circular DNA (rcDNA) and single-stranded DNA (ssDNA) [6,7]. To reduce the false positives, methods has been improved by pre-treating samples with Plasmid-safe ATP-dependent Dnase (PsD), which digests unmatured rcDNA. Recently, some studies have reported that sample treatment with T5 and several exonuclease may possess higher specificity than PsD treatment. Exonuclease T5 (ExoT5) digests ssDNA, rcDNA and double-strand

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linear DNA (ds DNA) [8,12]. In the present study, we used Exo I & III and developed a highly specific and sensitive method to quantify cccDNA.

Methods

Patients

During October 2014 to August 2016, 10 serum samples and 20 liver biopsy specimen from treatment-naïve CHB patients with viral load ($>10^5$ IU/mL) had been collected from Huashan Hospital, Fudan University. All the patients were tested negative for other hepadnavirus infection. Serum and liver tissue samples were immediately stored at −70 °C until further process (within 24 months). All the patients were recruited upon obtaining written informed consents.

Cell culture

HepAD38 cell line (ImQuestBioSciences, Frederick, MD, USA) were cultured in modified Eagle's medium (MEM)-F12 medium (Thermo Fisher, Waltham, MA, USA) supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin. The cells were seeded onto 12-well plates and cultured for approximate 4 days until the cell confluence reached more than 96%.

Mouse model

A specialized cloning vector was used in our study, and this vector can be removed through DNA recombination inside the transfected cells. For construction of the precursor plasmid prccccDNA, the chimeric intron was inserted into a circular HBV genome, with the single site replaced by two directly repeated segments flanking a prokaryotic plasmid backbone. Thus, a 3.3 kb rccccDNA bearing a chimeric intron can be produced from prccccDNA in the nuclei of hepatocytes. PrccccDNA (4–16 μg) eluted in a volume of phosphate-buffered saline (PBS) equivalent to 0.8% of the mouse's weight was injected through tail veins [13].

HBV cccDNA extraction

HBV cccDNA was isolated from biopsy specimen and other types of samples using hirt extraction. The samples were lysed in 1 mL SDS lysis buffer [50 mmol/L Tris–HCl (pH 8.0), 10 mmol/L EDTA, 150 mmol/L NaCl, and 1% SDS]. After 10 min incubation at room temperature, the lysates were transferred to a 1.5 mL microcentrifuge tube, mixed with 0.3 mL of 2.5 mol/L KCl, and incubated at 4 °C overnight with gentle agitation. After being centrifuged at 12,000 g for 25 min, the supernatant was extracted three times with phenol and chloroform. The DNA was extracted with ethanol, and washed three times with 70% ethanol, vacuum dried, and resuspended in TE [10 mmol/L Tris–HCl (pH 8.0)].

Serum HBV DNA was extracted by the QIAamp DNA Blood Mini Kit (Qiagen, Germantown, Maryland, USA) according to the manufacturer's instructions, and eluted into a final elution volume of 100 μL. HBV DNA was measured by the Cobas Amplicor HBV Monitor Test (Roche, Basel, Switzerland).

Enzymatic treatment

For Exo I & III (Thermo Fisher, Waltham, Massachusetts, USA) digestion, 25 μL HBV DNA prepared as described above was treated with 5 units of Exo I and 25 units of Exo III at 37 °C for 3 h in a total volume of 30 μL. For ExoT5 digestion, 25 μL DNA sample was treated with 5 units Exo T5 in a total volume of 30 μL at 37 °C for 3 h. And for PsD digestion, 25 μL DNA was treated with 20 units of PsD (Epicentre, Chicago, Illinois, USA) at 37 °C for 1 h in a total volume of 30 μL. The mixture was purified using Gel Band Purification Kit (Qiagen), and eluted in 30 μL of sterile double distilled water for the detection of HBV cccDNA using Roche Light Cycler 480 (Roche).

Control and quantification standards

Serum containing more than 10^8 IU/mL HBV DNA were filtered with 0.45 μm filters. Sucrose solution (30%) was filtered through 0.22 μm filters and then added to centrifuge tubes. The filtered serum was then slowly added onto the surface of sucrose solution (volume ratio of 2:1) and centrifuged at 25,000 rpm at 4 °C for 16 h. Supernatant was discarded and the pellet was dissolved with 200 μL of PBS at 4 °C overnight.

HBV DNA was isolated from serum of treatment-naïve CHB patients, and then the HBV whole genome plasmid pUC-536,207 (GenBank accession number AY220698, genotype B) was constructed as a positive control. Considering amplification efficiency, the lengths of the fragments should be consistent with cccDNA. The pcDNA3.1-HBV1.3 recombinant plasmid was digested into a haploid by restriction enzyme digestion and then ligated into a looped 3.2 kb DNA fragment as a quantitative standard.

Primer and probe design for cccDNA

To enhance sensitivity, primers were designed from conserved sequences in genotype A to H, and A conserved sequence was obtained by aligning the genotypes A–H, using DNAMAN program (Lynnon Biosoft, San Ramon, California, USA). This sequence was used to design PCR primers and probes. Primers and probes are listed in Table 1. Primers and probes were designed using Beacon Designer 8.0 (PREMIER Biosoft, California, USA). The primers and probes were synthesized by Invitrogen (Thermo Fisher, Shanghai, China).

PCR amplification

A duplicate real-time PCR was established. Primers and probes targeting a conserved region within the HBV gene for the quantification of cccDNA were designed. Primers for cccDNA targeting the gap region of the HBV genome were changed. The TaqMan probe was labeled with FAM. β-globin quantification specifically targeting the human gene with a HEX labeled probe were designed. Sequences of primers and probes are listed in Table 1 [14]. Fifty μL LightCycler 480 Master reactions (TakaRa, China) contained primers and probes. Five μL of DNA were added to 45 μL of the reaction premix. Real-time PCR was performed on Roche LightCycler 480 II platform using initial denaturation at 94 °C for 5 min followed by 40 cycles consisting of following steps: 10 s at 94 °C, 35 s at 60 °C, and 30 s at 72 °C. Fluorescence was measured in 2 channels (excitation-emission filters: FAM 478–520, HEX 525–563) at the end of each cycle at 72 °C for 30 s.

Statistical analysis

Statistical analysis was performed using SPSS 16 (SPSS Inc., Chicago, Illinois, USA). *P* values less than 0.05 were considered statistically significant.

Results

Sensitivity

The lower limits of detection were tested to be 10 copies/mL, since diluents containing HBV DNA less than 10 copies/mL produced inconsistent negative results. Based on the range of

Table 1
Primers and probes used in this study.

Primers and probes	Sequence	Length of product
cccDNA specific primers	5′-TCCCCGTCTGTGCCTTC-3′ 5′-CCCCAAAGCCACCCAA-3′	351bp
cccDNA specific probe	5′-FAM-TCT GCCGG ACCGT GTG-TAMARA-3′	
cccDNA non-specific primers	5′-GCAACTTTTTCACCTCTGCCTA-3′ 5′-AGTAACTCCACAGTAGCTCCAAATT-3′	139bp
cccDNA non-specific probe	5′-HEX-TTCAAGCCTCCAAGCTGTGCCTTGGGTGGC-TAMARA-3′	
Zoulim method's primers	5′-CTCCCCGTCTGTGCCTTCT-3′ 5′-GCCCCAAAGCCACCCAAG-3′	355bp
Zoulim method's probe	5′-GTTCACGGTGGTCTCCATGCAACGT-FL-3′ and 5′-R640-AGGTGAAGCGAAGTGCACACGGACC-P-3′	
β-globin primers	5′-GTGCACCTGACTCCTGAGGAGA-3′ 5′-CCTTGATACCAACCTGCCCAG-3′	121bp
β-globin probe	5′-HEX-AAGGTGAACGTGGATGAAGTTGGTGG-TAMARA-3′	

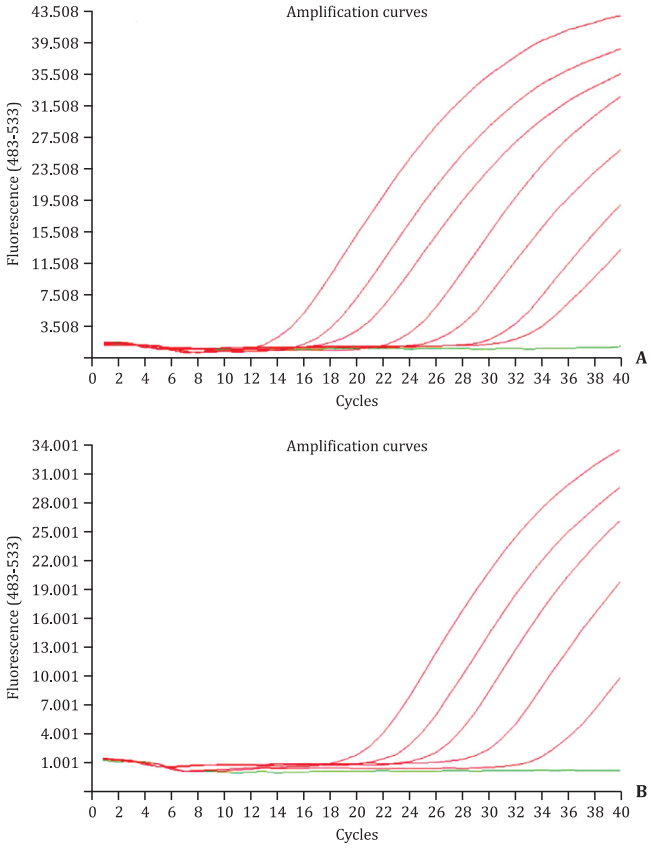

Fig. 1. Analytical sensitivity of the PCR assay for the detection of plasmid cccDNA. **A**: A line range of 1.0×10^1–1.0×10^7 was achieved using serial dilution of pcDNA3.1-HBV1.3 recombinant plasmid. **B**: Analytical sensitivity of the PCR assay for the detection of AD38 cellular cccDNA. A line range of 1.0×10^1–1.0×10^5 was achieved using serial dilution of AD38 cellular cccDNA.

detection, the Ct values and concentrations of the plasmids diluted 10-fold from 10^7 to 10^1 copies/mL were used to construct a standard curve (Fig. 1).

Specificity with enzymatic digestion

It was reported that PsD can be used to degrade rcDNA to enhance specificity of cccDNA detection. However, mature rcDNA cannot be degraded by PsD [15]. ExoT5 can be used to degrade rcDNA, dsDNA and ssDNA, but it can also degrade cccDNA intermediates. Fortunately, Exo I & III was reported to be able to digest all forms of HBV DNA except cccDNA and its intermediates [13]. Thus, we treated HBV cccDNA extracted from HepAD38 cells with either

Table 2
Effect of Exo I&III treatment on cccDNA-specific detection in serum samples.

No.	Cobas HBV DNA	Untreated HBV cccDNA	Exo I & III treated cccDNA
1	8.12E + 05	0	0
2	4.65E + 06	0	0
3	6.26E + 06	0	0
4	8.70E + 06	0	0
5	2.50E + 07	0	0
6	6.38E + 07	8.20E + 01	0
7	8.75E + 07	1.02E + 02	0
8	1.50E + 08	5.72E + 03	0
9	1.65E + 08	1.24E + 03	0
10	1.70E + 08	2.78E + 02	0

Exo I & III or PsD. The mean amplified log copy were 5.65, 4.57 and 3.46 in different amounts of HepAD38 cellular DNA templates (1000, 100 and 10 ng). After DNA were treated by Exo I & III and PsD, the mean amplified log copy number were 3.29, 2.34, 1.3 and 5.57, 4.49, 3.38, respectively. The levels of the PCR signals were decreased several folds following Exo I & III treatment, but the levels of the PCR signals are almost unchanged by PsD (Fig. 2A). cccDNA levels were measured in serum, and the results were shown in Table 2. These samples were tested after Exo I & III digestion which serum HBV DNA ranged from 8.12×10^5 to 1.70×10^8 copies/mL, all samples were found negative for cccDNA, and some samples with copies below 10^7 were also negative for cccDNA.

In addition, different amount of mouse model DNA (1000, 100 or 10 ng) templates with either Exo I & III or PsD treated were amplified by real-time PCR. The mean amplified log copy number of mouse model DNA templates of 1000, 100 and 10 ng input were 2.80, 1.90 and 0.91 after Exo I & III treatment; but the levels of cccDNA are almost unchanged by PsD (Fig. 2B).

Methods comparison

To evaluate specificity of amplification of HBV cccDNA, we compared the cccDNA specific primers which can specifically amplify cccDNA effectively versus a non-specific primer which can amplify both cccDNA and rcDNA as depicted in Table 1. In addition, Werle-Lapostolle et al. established a cccDNA detection methods which is widely cited by other researchers [14]. We used three methods to detect cccDNA in HepAD38 cells. Our method is almost identical to Zoulim's method for detecting cccDNA in HepAD38 cells, but non-specific primer for detecting cccDNA were promoted several folds, which means that a large amount of non-cccDNA were amplified (Fig. 2C).

Liver samples studies

Total HBV DNA and cccDNA levels were measured in liver biopsies (Table 3). The results of β-globin DNA were normalized

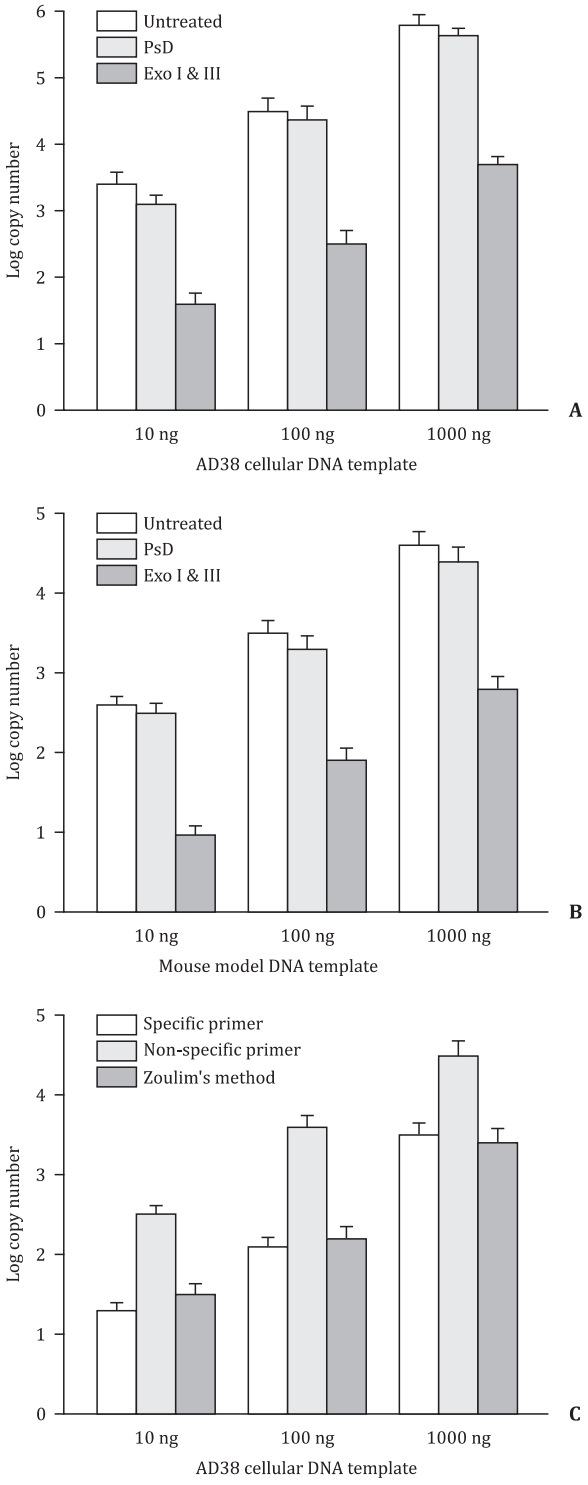

Fig. 2. **A**: Specific detection of cccDNA by qPCR following Exo I&III and PsD treatment in HepAD38 cells. **B**: Specific detection of cccDNA by qPCR following Exo I & III and PsD treatment in mouse model. **C**: Specific detection of cccDNA by different methods in HepAD38 cells.

to 1×10^6 cells. Total HBV DNA levels ranged from 2.37×10^6 to 1.70×10^8 per 1×10^6 cells, and cccDNA detected in all liver samples ranged from 1.56×10^4 to 9.79×10^5 per 1×10^6 cells, representing a significant proportion of total intrahepatic HBV DNA, 1.35% on the average (range 0.33%–2.60%). Intrahepatic HBV cccDNA levels correlated with levels of intrahepatic total HBV DNA.

Table 3
Specific detection of HBV total DNA and cccDNA in biopsy samples.

No.	Total HBV DNA	HBV cccDNA	cccDNA (%)
1	6.80E + 07	3.49E + 05	0.51
2	5.45E + 07	9.79E + 05	1.80
3	6.56E + 07	4.61E + 05	0.70
4	1.70E + 08	8.25E + 05	0.49
5	6.91E + 07	3.65E + 05	0.53
6	3.20E + 07	2.81E + 05	0.88
7	2.37E + 06	4.93E + 04	2.08
8	7.64E + 06	6.91E + 04	0.90
9	3.68E + 07	1.36E + 05	0.37
10	4.67E + 06	1.56E + 04	0.37
11	2.38E + 07	3.49E + 05	0.33
12	6.55E + 07	3.79E + 05	1.47
13	2.35E + 07	6.10E + 05	0.58
14	1.28E + 08	9.50E + 05	2.60
15	3.51E + 07	6.80E + 05	0.74
16	7.92E + 07	7.10E + 05	1.94
17	5.37E + 06	2.90E + 04	0.90
18	5.47E + 06	7.10E + 04	0.54
19	3.98E + 07	1.76E + 05	1.30
20	3.87E + 07	2.46E + 05	0.44

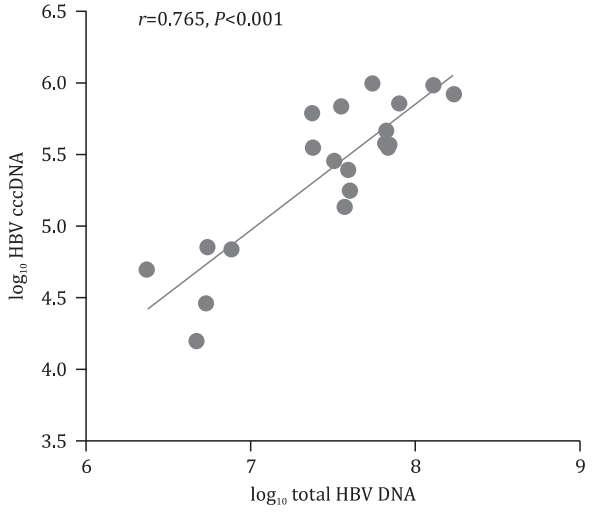

Fig. 3. Correlation between intrahepatic HBV cccDNA and total HBV DNA in liver tissue sample.

The correlation of intrahepatic cccDNA and HBV tDNA was significant ($r = 0.765$, $P < 0.001$) (Fig. 3).

Discussion

Considering the role of cccDNA in HBV life cycle, it can serve as an indicator for replication and persistence status of the virus. Monitoring cccDNA during and after antiviral therapy is essential for routine treatment of chronic hepatitis B [15–18]. The cccDNA fluctuations in antiviral therapy may reflect either the effectiveness of antiretroviral drug or the resistance to the therapy. The main concern in cccDNA design is the complexity of the HBV genome structure, which may affect the non-specific background signal. Some reports have been used to quantify viral cccDNA. Chen et al. [19] established a real-time quantitative PCR method to detect cccDNA. Shao et al. [20] created cccDNA detective method based on chimeric primers and real-time fluorescent PCR. Wong et al. [21] established Invitations Assay of cccDNA. However, all of these methods elaborate false positives and have no strict control measures. cccDNA clinical test is extremely restricted. There are three difficulties in the HBV cccDNA detection. The first one is the large number of other forms of HBV DNA with high homology to

the cccDNA sequence, including rcDNA, ds-DNA, pregenomic RNA (pgRNA), ssDNA and so on. Second, it is clear that only 5–10 copies of cccDNA are present in each hepatic cell, and cccDNA accounts only a very small portion of the total number of HBV DNA in the cell. Some study showed that the total HBV DNA was 10.7–92,000 fold higher than that of cccDNA [16–18]. Third, HBV cccDNA is only present in the nucleus of infected cells, and the presence of HBV cccDNA has not been observed in the cytoplasm. Therefore, it is more difficult to determine whether HBV cccDNA enters the cell through the cell membrane.

In this study, we developed a highly specific and sensitive method to detect the HBV cccDNA. We developed the real-time PCR method for cccDNA based on several factors, including the small size PCR product and the specificity of the primers. The PCR amplification product was designed to be less than 400 bp, which ensures efficient amplification from low levels of target DNA. The specificity of HBV cccDNA detection is based on two important factors: (a) the digestion of non-cccDNA by Exo I & III for removal of interfering DNAs such as ssDNA, rcDNA and so on; (b) preferential amplification of cccDNA by specific primers that located on both sides of the gap of rcDNA. The use of multiple dyes at different emission wave lengths will allow simultaneous detection of internal control in the same tube. The detection method is fast and specific. Detection of specific products needs 40 cycles with an average of 60 min.

The analytic sensitivity was also tested using serially diluted HBV plasmid DNA. Our study found that fluorescence signal could be detected when PCR runs 40 cycles for the low template concentration (1.0×10^1 copies of plasmid DNA). In regular methods, increasing sensitivity may reduce specificity and vice versa [22,23]. Our study has achieved a balance between specificity and sensitivity. In addition, intrahepatic HBV cccDNA level had a good correlation with intrahepatic total HBV DNA level. The possible explanation is that HBV cccDNA levels are closely associated with persistent HBV infection that could not be completely reflected by serum virologic and serologic markers [24,25].

We used Exo I & III to remove any DNA but cccDNA. The specificity of cccDNA was confirmed by the absence of amplification when Exo I & III digested serum samples, in addition, when without Exo I & III digested treatment, 5 of the 10 high-dose were detected for non-specific cccDNA in hepatitis B virus serum samples. Some studies failed to detect cccDNA in serum [8,18]. Although other investigators have reported the presence of HBV cccDNA in the blood stream, it may not rule out non-specific cccDNA. Pretreatment of Exo I & III is necessary, since non-specific amplification is significantly increased without Exo I & III treatment. Exo I & III treatment can minimize the impact of HBV rcDNA and improve the detection of HBV cccDNA specificity. In addition, we used HepAD38 cells and the recombinant gene of the total cccDNA mouse model to verify the specificity of the protocol, and achieve the desired results.

In order to reduce false positives, we set a rigorous control. We isolated the virus particles from peripheral blood mononuclear cells as a negative control. Considering the consistency of amplification efficiency and the authenticity of the expanded environment, we used the recombinant 3.2 kb circular DNA fragment to simulate cccDNA as our standard and positive control [12]. With this design, we maximize the consistency of cccDNA amplification in vitro and in vivo.

In summary, our study provides an innovative approach to detect HBV cccDNA. In this study, we used plasmids, cell lines, mouse model, serum samples, and tissue samples to systematically evaluate the specificity and sensitivity of cccDNA assay. The method has a high sensitivity, good reproducibility, high specificity and high accuracy. Our novel and selective detection of cccDNA is a useful tool for monitoring the effect of antiviral therapy and other potentially important pathogenesis problems. Our data may be applicable in clinical practice.

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Contributors

ZJM conceived the study. JPX prepared the study protocol and drafted the manuscript. MRC, DMH and YXP collected and analyzed the data. XQ, WJY, QD and ZJM performed the experiments. All authors approved the final version of the manuscript. ZJM is the guarantor.

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Ethical approval

This study was approved by the Institutional Ethics Committee of Huashan Hospital, Fudan University, China (2015–0051).

Competing interest

No benefits in any form have been received or will be received from a commercial party related directly or indirectly to the subject of this article.

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