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Anne Boerekamps[1]
Thomas Vanwolleghem[2,3]
Marc van der Valk[4]
Guido E. van den Berk[5]
Marjo van Kasteren[6]
Dirk Posthouwer[7]
Anthonius S.M. Dofferhoff[8]
Bart van Hoek[9]
Dewkoemar Ramsoekh[10]
Jelle Koopsen[11]
Janke Schinkel[11]
Eric Florence[12]
Joop E. Arends[13]
Bart J. Rijnders[1,*]

[1]*Department of Internal Medicine and Infectious Diseases, Erasmus MC, Rotterdam, the Netherlands*
[2]*Department of Gastroenterology and Hepatology, University Hospital Antwerp, Antwerp, Belgium*
[3]*Department of Gastroenterology and Hepatology, Erasmus MC, Rotterdam, the Netherlands*
[4]*Division of Infectious Diseases, Amsterdam Infection and Immunity Institute, Academic Medical Center, Amsterdam, the Netherlands*
[5]*Department of Internal Medicine and Infectious Diseases, OLVG, Amsterdam, the Netherlands*
[6]*Department of Internal Medicine and Infectious Diseases, Elisabeth-TweeSteden Ziekenhuis, Tilburg, the Netherlands*
[7]*Department of Internal Medicine and Medical Microbiology, Maastricht Universitair Medisch Centrum+, Maastricht, the Netherlands*
[8]*Department of Internal Medicine and Infectious Diseases, Radboud University Medical Center, Nijmegen, the Netherlands*
[9]*Department of Gastroenterology and Hepatology, Leiden University Medical Center, Leiden, the Netherlands*
[10]*Department of Gastroenterology and Hepatology, VU University Medical Center, Amsterdam, the Netherlands*
[11]*Department of Medical Microbiology, Section of Clinical Virology, Academic Medical Center, Amsterdam, the Netherlands*
[12]*Department of Clinical Sciences, Institute of Tropical Medicine, Antwerp, Belgium*
[13]*Department of Internal Medicine and Infectious Diseases, Universitair Medisch Centrum Utrecht, Utrecht University, Utrecht, the Netherlands*
*Corresponding author. Address: Department of Internal Medicine and Infectious Diseases, Erasmus MC, PO Box 2040, 3000 CA Rotterdam, the Netherlands.
E-mail address: bartrijnders@me.com (B.J. Rijnders).

Over-gap PCR amplification to identify presence of replication-competent HBV DNA from integrated HBV DNA: An updated occult HBV infection definition

To the Editor:

With great interest, we read the manuscript "Quantitation of HBV cccDNA in anti-HBc-positive liver donors by droplet digital PCR: a new tool to detect occult infection" by Caviglia *et al.* published in *Journal of Hepatology*.[1] Using a highly sensitive in-house droplet digital PCR assay (ddPCR) method, the authors indicated that intrahepatic HBV covalently closed circular (cccDNA) was detectable in about half (52%, 27/52) of the defined cases of occult HBV infection (OBI). We wonder whether the pretreatment with plasmid-safe ATP dependent DNase (PSAD) plus double-over-gap cccDNA 'specific' primers spanning the HBV relaxed circular DNA (rcDNA) gap region used in this paper could totally eliminate the interference of rcDNA, though this method had been widely used in the detection of cccDNA.[2] Here we evaluated the capacity of the above approach to discriminate between the cccDNA, the rcDNA and the integrated double strand linear HBV DNA (dslDNA). In addition, several sets of mono-over-gap rcDNA primers (Table S1) were also tested, which theoretically can amplify both rcDNA and cccDNA.[3]

First, to exclude the likely cccDNA contaminant leaked from cells, the supernatant of HepAD38[2] and serum specimens from patients with HBV infection[4] were treated with DNase I prior to viral DNA extraction and PCR amplification. The elimination efficiency was confirmed by the failed amplification of plasmid DNA containing 1.2xHBV genome (Fig. 1A). In contrast, the HBV rcDNA in Dane particles could still be detected by using the supposed cccDNA 'specific' primers, which provided a similar result compared to rcDNA primers. Moreover, the gradual increase of HBV DNA level was observed in parallel with the increased amount of rcDNA, when either the supposed cccDNA primers or the rcDNA primers were used (Fig. 1B). As previously reported,[5] the rcDNA could not be eliminated completely pretreatment by PSAD and this was further confirmed by T5 Exonuclease and Exonuclease III, respectively (Fig. 1C, D). Hence, PSAD digestion plus double-over-gap PCR may not guarantee the discrimination of cccDNA from rcDNA.

The term 'occult hepatitis B virus infection' has been introduced to describe a status characterized as an absence of serum HBV surface antigen and presence of replication-competent HBV DNA in the liver.[6–8] Since cccDNA is the resource for viral replication and the reason for HBV infection persistence, the presence of cccDNA for OBI is indispensable. The integrated HBV DNA fragments, on the other hand, have an incomplete viral genome which lost the capacity to serve as the template for HBV replication. Therefore, it is reasonable to postulate that the detection of cccDNA, but not the presence of integrated HBV DNA fragment, is essential for true OBI. Moreover, it may not be necessary to distinguish cccDNA from rcDNA for the definition of OBI because the rcDNA originates solely from the transcriptionally active cccDNA.[9]

Integration of HBV DNA fragments is a common event during HBV infection. Our previous study revealed that the breakpoints of the integrated HBV DNA fragments were mainly found within the DR1 and DR2 regions (Fig. 1E).[4] This is in accordance with the suggestion that HBV dslDNA is the preferred form for viral DNA integration into the host genome.[10] To test if the integrated

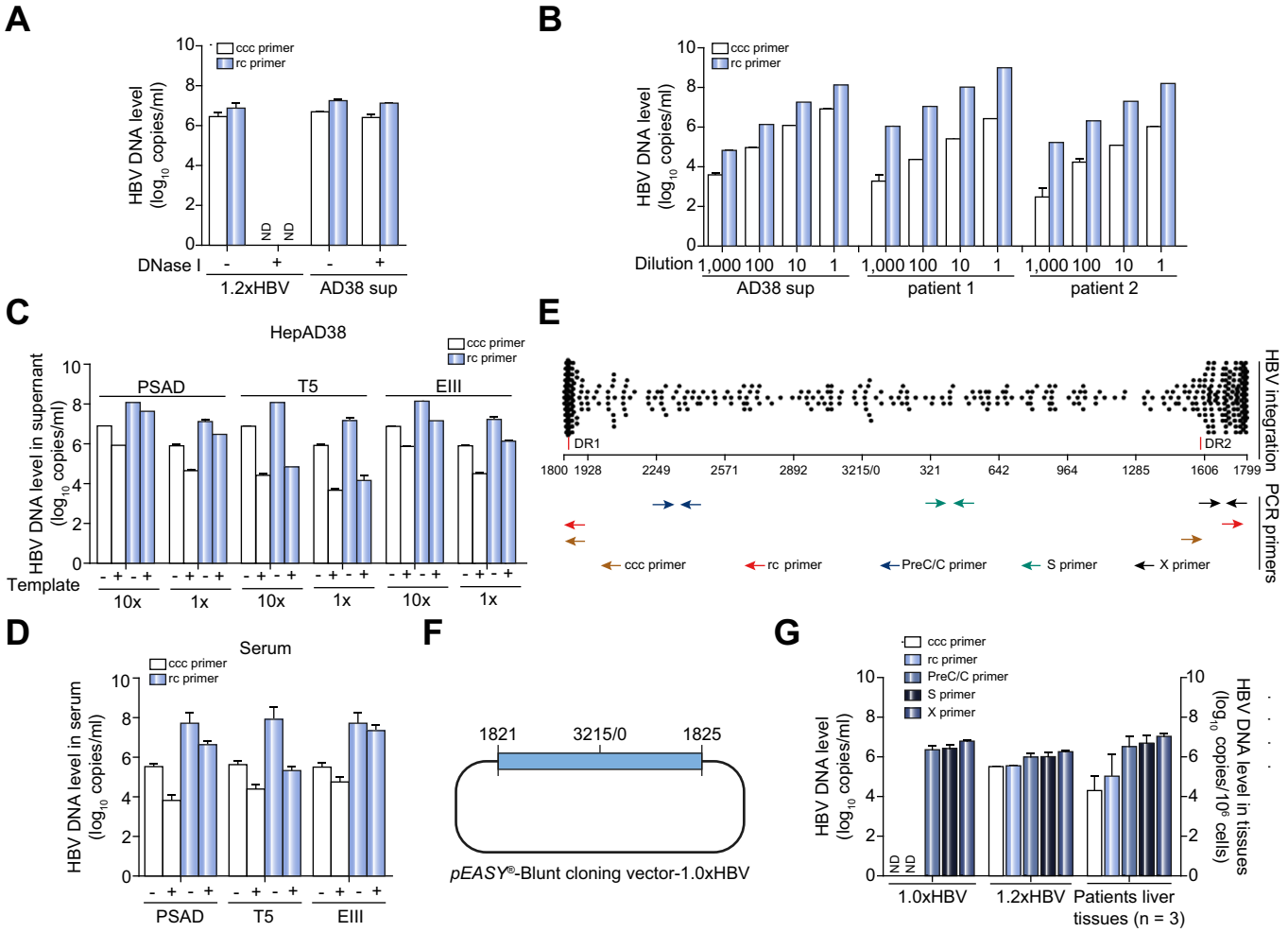

Fig. 1. Analysis of the characteristics of HBV over-gap PCR detection and its capacity to discriminate between cccDNA, rcDNA and integrated HBV DNA. (A) The PCR amplification of supposed cccDNA primers (double-over-gap) and rcDNA primers (mono-over-gap) with the 1.2xHBV plasmid and the supernatant of HepAD38 pretreated with DNase I. AD38 sup, HepAD38 supernatant. ND, undetectable. (B) The consistent dynamic tendency of PCR amplification between cccDNA and rcDNA primers using the supernatant of HepAD38 and serum from patients pretreated with DNase I. The number under the x axis indicates the gradient dilution fold. AD38 sup, HepAD38 supernatant. (C, D) The PCR amplification of supposed cccDNA primers and rcDNA primers plus digestion with DNases using the template DNA from HepAD38 cell supernatant and serum from patients (n = 2). The samples had been pretreated with DNase I before viral DNA extraction and then digested by PSAD, T5 and EIII, respectively. The "+" indicates the samples were treated with the enzyme while the "−" means parallel control without the enzyme. The "10×" and "1×" indicate the input sample volume in the PSAD, T5 and EIII digestion system, respectively. (E) The distribution of HBV integration breakpoints and PCR primers on viral genome. The dots represent HBV integration breakpoints identified in liver biopsies of patients with HBV infection. The HBV genome was lined and scaled from 1,800 to 1,799 nt and the DR regions were indicated by rectangles in red. (F) The schematic diagram of 1.0 × HBV plasmid carrying full-length HBV genome ranging from 1,821 to 1,825. (G) The PCR amplification of cccDNA and rcDNA with double-over-gap and mono-over-gap primers respectively from the 1.0 × HBV construct mimicking HBV integration, the 1.2xHBV plasmid and liver tissues (n = 3) from patients with HBV infection. The PCR amplification targeting HBV genome regions of PreC/C, S, and X was also performed as a control. For the HBV DNA level in liver tissues, the DNA copy number was normalized to cell number according to the input DNA mass and the estimated DNA mass per cell. cccDNA, covalently closed circular DNA; rcDNA, relaxed circular DNA; DR, direct repeat; EIII, Exonuclease III; ND, undetectable; PSAD, plasmid-safe ATP dependent DNase; T5, T5 Exonuclease.

HBV DNA could interfere with the amplification specificity of cccDNA or rcDNA primers, a 1.0 × HBV plasmid carrying the full-length HBV genome ranging from 1,821 to 1,825 was constructed to mimic the HBV integration (Fig. 1F). Since the orientation of cccDNA and/or rcDNA paired primers were outward to each other, PCR amplification would be invalid when using 1.0xHBV plasmid as template. As expected, neither cccDNA nor rcDNA primers had valid PCR amplification. In contrast, successful PCR reactions were observed with the 1.2 × HBV plasmid, which could mimic cccDNA from the liver tissues of patients with chronic HBV.[4] Nevertheless, the other three primer sets (Table S2) specific to PreC/C, S and X regions could amplify all three kinds of templates (Fig. 1G). Taken together, these results indicated mono- or double-over-gap PCR primers could distinguish cccDNA and rcDNA from integrated HBV DNA.

In summary, we proposed the absence of serum HBV surface antigen and the presence of HBV rcDNA and/or cccDNA as the true OBI. The mono- or double-over-gap PCR primers could specifically amplify the rcDNA and/or cccDNA but not the integrated DNA, which could be used in laboratory-based identification for OBI in the future.

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Conflict of interest

The authors declare no conflicts of interest that pertain to this work.

Please refer to the accompanying ICMJE disclosure forms for further details.

Authors' contributions

Lu F, Liu Y, Liao H designed the research. Liu Y, Zeng W, Xi J, Liu H, Liao H and Yu G performed the research. All authors analyzed the data. Liu Y wrote the paper. Lu F and Chen X revised the paper.

Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jhep.2018.10.003.

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Author names in bold designate shared co-first authorship

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Yongzhen Liu
Wanjia Zeng
Jingyuan Xi
Hui Liu
Hao Liao
Guangxin Yu
Xiangmei Chen
Fengmin Lu*

State Key Laboratory of Natural and Biomimetic Drugs, Department of Microbiology & Infectious Disease Center, School of Basic Medical Sciences, Peking University Health Science Center, Beijing 100191, PR China

*Corresponding author. Address: State Key Laboratory of Natural and Biomimetic Drugs, Department of Microbiology & Infectious Disease Center, School of Basic Medical Sciences, Peking University Health Science Center, 38 Xueyuan Road, Beijing 100191, PR China. Tel: +86 10 82805136, fax: +86 10 82805136.
E-mail address: lu.fengmin@hsc.pku.edu.cn

Reply to: "Over-gap PCR amplification to identify presence of replication-competent HBV DNA from integrated HBV DNA: An updated occult HBV infection definition"

To the Editor:

Recently, we developed a novel digital droplet PCR (ddPCR) assay for the selective quantitation of intrahepatic HBV covalently-closed-circular DNA (cccDNA), the plasmid-like episome form of HBV DNA that serves as template for all viral RNAs.[1] We used this method to determine the prevalence and quantity of HBV cccDNA in individuals with occult HBV infection (OBI) recruited among a cohort of 100 hepatitis B surface (HBsAg)-negative/antibody to hepatitis B core antigen (anti-HBc)-positive liver donors. A total of 52 donors were found to have OBI (*i.e.* presence of total HBV DNA in the liver in the absence of detectable HBsAg),[2] while HBV cccDNA was detected in 27 (52%) of the OBI-positive individuals, with a median of 13 (5–25) copies/10^5 cells.

We thank Dr Liu and colleagues for their interesting data and comments.[3] To investigate the efficacy of different nucleases on the selective removal of relaxed circular HBV DNA (rcDNA) and integrated double strand linear HBV DNA (dslDNA) from HBV cccDNA and to assess the specificity for HBV cccDNA amplification of double-over-gap primers, supernatant of HepAD38 (cells expressing proteins, RNAs and DNA intermediates of HBV) and serum specimens of patients with chronic HBV infection were differentially treated with plasmid-safe ATP dependent DNase (PSAD), T5 exonulease (T5 Exo) and exonuclease III (Exo III). Then, digested DNA samples were amplified by quantitative PCR (qPCR) using either double-over-gap specific primers for HBV cccDNA or mono-over-gap rcDNA primers. Authors observed that no nuclease was able to completely remove rcDNA; moreover, double-over-gap primers did not show absolute specificity for HBV cccDNA.

In response to Liu *et al.*, we would underline that both our method and the samples tested were partly different from those described by the authors. Firstly, we developed a ddPCR assay based on fluorescent-probes characterized by a higher target-specificity in comparison to a qPCR assay using DNA-specific intercalation dye. Secondly, some concerns may arise regarding the specificity of qPCR amplification since no negative control can be observed through all the reported experiments. Lastly,