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Conflict of interest

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

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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

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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,

liver DNA extracts from HBsAg-negative/anti-HBc-positive liver donors are substantially different from supernatant of cellular-lines and serum samples from patients with chronic hepatitis B.

In studies aiming to quantify intrahepatic HBV cccDNA, the key point is the selective detection of supercoiled DNA in the presence of a complex background consisting of genomic DNA, viral rcDNA and other replicative intermediates. To our knowledge, most of the studies performed so far, adopted a PSAD-based purification method in association with specific primers spanning the gap region of rcDNA.[4–7] In agreement with Liu and colleagues, as well as with previous studies,[8] we observed that the use of selective primers without enzymatic digestion may not guarantee an absolute specificity for HBV cccDNA amplification, especially in the presence of a high excess of rcDNA. In addition, which nuclease should be preferred to enrich HBV cccDNA for further PCR analysis is still debated. In this regard, Qu and colleagues recently investigated the efficacy of different nucleases, including PSAD, T5 Exo and Exo III, for selective removal of rcDNA without degradation of HBV cccDNA by using total cell lysates from *in vitro* infected cells;[9] despite T5 Exo showing a certain degree of activity towards cccDNA when a longer incubation time was adopted, the enzyme was more efficient for rcDNA, open circular DNA and genomic DNA removal for *in vitro* assays.

Another important aspect is that experimental conditions for PCR quantitation of HBV cccDNA may vary according to the type of samples (*i.e.* liver samples, serum samples, cell cultures, *etc.*). In our series,[1] we did not perform a head-to-head comparison between PSAD, T5 Exo and Exo III; however, considering that rcDNA contamination in the liver of OBI-positive individuals may be negligible, our ddPCR-based assay appears, at present, reliable for directly determining HBV cccDNA in liver specimens of individuals with markers of previous HBV exposure but no history of liver disease.

Finally, Liu and colleagues proposed to improve the current definition of OBI, "absence of serum HBsAg and the presence of HBV rcDNA and/or cccDNA" in the liver,[3] based on the postulate that HBV cccDNA is the replication-competent form of the virus from which originates rcDNA, while integrated dslDNA is characterized only by fragments of HBV DNA without the capacity to give rise to novel viral progeny.[10] On this point, we think that OBI definition may be refined to consider both novel virological/immunological concepts and recent technical improvements that, in the near future, will allow a more precise identification of the different HBV DNA species in the liver.

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Drafting the manuscript: Gian Paolo Caviglia, Antonella Olivero.

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Supplementary data

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