Reviewer's report

Title: A novel method for detection of HBVcccDNA in hepatocytes using rolling circle amplification combined with in situ PCR

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Reviewer: Scott Bowden

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Hepatitis B virus (HBV) infection is initiated by the formation of HBV covalently closed circular (ccc) DNA, the key HBV intrahepatic replicative intermediate, in the hepatocyte nucleus. There, HBV ccc DNA associated with host cell histones to form a stable minichromosome which is largely refractory to elimination by the host immune response or antiviral therapy. Several PCR-based assays have been developed for detection and quantification of intrahepatic HBV ccc DNA but interpretation can be problematic. HBV ccc DNA assays lack standardization, sensitivity can be low and specificity poor due to non-specific amplification. Some of these difficulties have also plagued assays for the in situ detection of HBV ccc DNA in liver tissue.

In this manuscript, the authors overcome many of these obstacles by developing a technique combining two target amplification technologies, rolling circle amplification (RCA) and in situ PCR. Specificity has been enhanced by the use of Plasmid-Safe DNase and the use of multiple primer binding sites for the RCA step. The digoxigenin staining in the hepatocyte nuclei at the higher magnification provides convincing evidence of the presence of HBV ccc DNA.

I believe there are some improvements the authors can make to strengthen the report and these are described below.

Major
1. The authors do need to enlist the assistance of a colleague with good English skills to edit grammar, typographical and punctuation errors. As an example, if one looks at the last few sentences of the Background, it should read: “In a previous study, we developed a quantification assay by introducing a rolling circle amplification (RCA) step combined with plasmid-safe ATP-dependent DNase (PSAD) digestion and real-time PCR, which offered better resolution [14]. However, the quantitative …”

Minor
2. Table 1 does contain some HBV DNA load data, so details of which assay was used should be described in Materials and Methods.
3. The authors claim that the sensitivity of their test is two copies/cell but no evidence is provided as to how this was deduced.
4. The sub-heading RCA treatment would be better described as RCA assay.
5. Similarly, the heading Evaluation of sensitivity, repeatability and specificity of the assay would be better having reproducibility in place of repeatability.

6. In the last paragraph of the Discussion, I am sure the authors do no mean “an electron microscope” but rather a conventional light microscope.

Discretionary

7. I do not believe that the Figure headings are an apt description as it is not standard immunochemistry staining.

8. The positive HBV ccc DNA staining is difficult to recognize at the x200 magnification and this will be further exacerbated when the figures are reduced on publication. They authors may wish to consider only including the x400 magnification which ably illustrates their results.

Level of interest: An article of importance in its field

Quality of written English: Needs some language corrections before being published

Statistical review: No, the manuscript does not need to be seen by a statistician.

Declaration of competing interests: none