Reviewer's report

Title: Comparison of Sanger sequencing for Hepatitis C Virus Genotyping with a Commercial Line Probe Assay in a tertiary hospital

Version: 0 Date: 10 Oct 2018

Reviewer: Lize Cuypers

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The authors present an alternative to the commercially available assays to determine HCV genotypes and subtypes as misclassifications have been reported in a substantial number of cases. Either in case of failure in the determination of the HCV genotype or when distinguishing between the HCV subtypes of genotype 1 by the LiPA assay, sequencing the 5'UTR, the core and the NS5B region proved to be able to classify these cases. A sequencing assay based on LiPA amplicons for 5'UTR and core, complemented with the amplification of a fragment within NS5B, to determine the HCV genotype and subtype in case of failure by LiPA, can be valuable in clinical laboratories to resolve misclassifications, as these can highly influence the choice of a proper treatment regimen.

Major comments:

1. The authors present their method as being highly innovative, while I do not agree with this statement. Re-using the UTR and core amplicons generated by the LiPA procedure is definitely a large step forward in the feasibility of performing sequencing assays for clinical laboratories to determine HCV genotypes and subtypes. However, to target the NS5B region, a separate assay starting from extraction should be followed in order to obtain these sequences. Sequencing a short and conserved stretch of the NS5B gene is common practice and has been published numerous times in the past, not providing any novelty to the current practice. Although I'm absolutely in favor of HCV genetic sequencing, I'm often confronted with the unwillingness of clinical laboratories to move towards sequencing assays. I believe the authors should elaborate more on the actual use of these assays in clinical practice, such as when is it advised and to provide an estimation of the % of cases in which they would recommend to perform additional testing. Moreover, as the NS5B amplification and sequencing starts from scratch, do the authors think it is feasible to always have enough plasma volume to perform this additional testing, what about the extra time and cost that this will require? Do they think the NS5B success rate of less than 90% is sufficient to implement in routine practice?

2. While the authors describe in the methods that several samples have been selected for sequencing using different genetic regions, it is not clear why this was done for certain samples
and not for all samples. I guess this might be an issue of sufficient sample volume, however this is not properly explained. Table 2 provides insight in which samples have been sequenced using more than one genetic region, and how the LiPA and sequencing assays compare. However, in the results the different genetic regions are discussed separately, only comparing LiPA results with the results of each sequencing assay separately (so gene per gene), not providing any clue for the reader whether results obtained between sequencing of 5'UTR, Core and/or NS5B were consistent or not. The current representation of the results is therefore quite confusing for readers, only Table 2 is currently of value. Please consider to re-organize Table 2. I would suggest to add a column for the final assignment of each sample and to organize the samples according to LiPA assignment (starting from 1a) instead of by sample number, as the latter has no meaning to readers. Highlights or colors to visualize whether results were consistent among assays would be very useful. Also explain the abbreviations more in-depth, NR (not realized, why?), NI (not interpretable, this does mean that an actual sequencing attempt was performed and why not interpretable? Low quality or ?) and PA = ?

3. Why do the authors determine the HCV genotype and subtype after sequencing only with the so-called HCV genomic blast bank? Of note, this is not how you should refer to the use of ncbi blast. Why don't they use a proper way of subtyping, such as constructing a phylogenetic tree? That way they might be able to distinguish between certain HCV4 subtypes for NS5B sequences (and especially when more than one genetic region was sequenced), as in Table 2 often more than one subtype was reported. Moreover, which threshold for similarity was used when performing a blast search? As you are using highly conserved regions across the HCV genome and moreover short sequences, this is of importance.

Minor comments:

1. How was the sample selection done? The authors mention that they wanted to cover the different HCV genotypes and subtypes that are prevalent in more than 2% of cases in Belgium, however they also state that the selection is not representable for the Belgian epidemic. First of all, can the authors provide more information on the Belgian epidemic in the introduction, and subsequently explain more in detail how these 100 samples were selected? A substantial high proportion of these samples, respectively 8 and 27, was not properly classified with LiPA, either not discriminating between G1 and 6, or being undetermined. Compared to the percentages that are reported throughout the paper, only for between 2-7% of samples LiPA is not able to determine the HCV1 subtype, so these 100 samples are not just a random set of samples, being not representative for what is generally seen in the clinic.

2. Please choose between a numerical value (23) or writing in full (twenty-three) instead of using both in the Abstract.

3. Introduction: please note that already more than 80 subtypes were identified.
4. Introduction: the authors give the impression that HCV genotype determination has become key since the approval of DAAs, however this has been always highly important to initiate treatment, already in the IFN era. I would suggest the authors to add a section in the introduction about the impact of HCV genotype misclassifications on the treatment of patients. There have been published several reports stating virological failure in patients due to misclassification of the HCV genotype and/or subtype, and therefore subsequently the wrong choice of treatment regimen.

5. The authors do not mention anything about mixed infections in the paper. Please elaborate on this aspect of HCV genotyping, as this is another issue associated to the use of LiPA.

6. The authors use Sanger population sequencing in their assay, however how long do they think it is going to be still used in clinical practice? What about NGS and its compatibility with the proposed method using LiPA amplicons? In general, the discussion is very limited.

7. Ethics: can the authors provide a study or approval number? Have the generated sequences been submitted to Genbank?

8. The amplification success rate for NS5B is rather low, the authors claim that it is comparable to what has been reported in one study. However, many other studies do much better, hence why a more in-depth discussion on this aspect is required. Can the authors identify other reasons why the amplification was not successful in more than 10% of samples, next to the somewhat higher genetic diversity compared to 5'UTR and Core? What about viral load? And have the samples been tested in duplicate or triplicate? The authors should be aware that far more diverse regions of the HCV genome have been successfully sequenced.

9. Conclusion: referring to a study using the Abbott assay while the entire manuscript compares the determination success of LiPA and sequencing based assays is not appropriate, this should be moved to the discussion section.
Are the methods appropriate and well described?
If not, please specify what is required in your comments to the authors.

No

Does the work include the necessary controls?
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Yes

Are the conclusions drawn adequately supported by the data shown?
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