Reviewer's report

Title: Development of real-time NASBA assays with molecular beacon detection to quantify mRNA coding for HHV-8 lytic and latent genes

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Reviewer: Dr Jaap M. Middeldorp

Level of interest: A paper of considerable general medical or scientific interest

Advice on publication: Unable to decide on acceptance or rejection until the authors have responded to the compulsory revisions

The paper of Polstra et al describes the development and use of 4 real-time NASBA assays for quantitative detection of 4 different HHV8 mRNA targets and the use of U1A mRNA quantification by NASBA for normalisation.

The described real-time NASBA assays appear an excellent analytical tools for detection and quantification of in vitro transcribed HHV8 RNA, as demonstrated in Figure 2.

1. However, the description of specificity should include a statement on the level of alternative cRNA targets used in interference studies (page 10, 2nd allinea).

2. No information is provided on the reliability of the methods in a clinical relevant sample background (e.g. total leukocyte DNA/RNA of different healthy donors and/or patients on HAART). Because different sample backgrounds and different treatment protocols may influence the efficiency of individual NASBA reactions, this is considered to be essential. Alternatively, the use of an internal standard construct simultaneously and competitively amplified and detected by a specific second molecular beacon is suggested for each NASBA.

3. Because (a) RNA transcriptional activity of the U1A gene may vary with the level of overall transcriptional activity (aEoeactivation stateaE) of leukocytes and (b) leukocyte activation may differ at different points during the disease process and (c) the relative quantity and activation stage of different leukocyte subpopulations in the circulation may vary during the follow-up period, normalisation of the NASBA results by using U1A RNA levels of the assay input may not be used as reliable parameter. Rather the total leukocyte RNA or even better the total leukocyte DNA input per assay should be standardised (equal input of total cells) in order to allow for correct standardization of the HHV8 RNA transcription results.

This may be the reason for the unexplained findings of unexpected variations in the specific HHV8 RNA transcription levels as shown in Table 3.

There is no explanation as to the meaning of +, ++, +++ for the levels of individual HHV8 RNA results presented in Table 3.
4. The paper would gain considerable relevance and publication value if the authors would include data on HHV8 transcription levels in random HIV carriers that are seropositive (and seronegative) for HHV8 and extend the HHV8 findings to more than the current 2 patients, including samples of KS patients at different stage of disease with known levels of HHV8 DNA.

5. The presentation of only two KS cases with rather variable results of HHV8 RNA levels and the lack of additional patient or healthy controls does not permit interpretation of the overall diagnostic relevance of the different HHV8 NASBA assays.

6. Some additional data from literature or some more detailed discussion relating to the nature of cells (leukocytes or KS cells) responsible for the HHV8 levels observed in blood leukocytes would add to the relevance of the discussion. The current discussion section is considered too limited.

In view of these points of criticism, the authors are advised to improve the manuscript in order to allow a justified publication via BioMed Central.

**Competing interests:**

None declared.