Peer Review File
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Reviewer A

Line 84: to replace the word "weapons" with a more appropriate word

- ANSWER: Thanks for this comment. “weapons” replaced with “tools”

Line 88: author mentioned "infected person". "Specimen" or "clinical sample" may be more appropriate.

- ANSWER: Thanks for this comment. “infected person” replaced with “clinical sample”

Line 91: Author mentioned "end point of measurement". This may cause confusion between real-time PCR and conventional end-point PCR.

- ANSWER: Good point, thanks. "end point of measurement" replaced with “final results”

Lines 93-95: definition of ct is misleading

- ANSWER: Thanks for this comment. Revised as follows: “In other words, a low Ct value reflects a high concentration of viral genetic material, while a high Ct value reflects a low concentration of viral genetic material”

Line 118": suggest to remove the word "even"

- ANSWER: Done, as suggested.

Lines 220-229: Prediction of emergence of new variants. New variants may escape of host immune system resulting in increased infectivity. However, genomic variants may results in mismatches of primers/probes binding sites of existing PCR-based assays due to suboptimal PCR amplification or PCR amplification failure. So increase in ct values is also a sign of emergence of mutant strains.

- ANSWER: Very good point, thanks. Concept now included, as follows “However, genomic variants can lead to mismatches of primers/probes binding sites of existing PCR-based assays due to suboptimal PCR amplification or PCR amplification failure. Therefore, an increase in Ct values may also be an alarming sign for the occurrence of mutant strains.”

Were all the data discussed in this review from studies on upper respiratory specimens- are there some data from lower respiratory specimens?

- ANSWER: This is true and has been clearly specified also in the title. We focused on upper respiratory specimens because this is the most frequent sample that can be collected from patients. Investigating Ct values in other samples has not been considered a focus of our study

There are other molecular technologies other than real-time reverse-transcription PCR providing viral load, one example is droplet digital PCR (ddPCR) which gives absolute count of viral copy number. Good to discuss.

- ANSWER: Thanks, for the suggestion, we have hence included the following sentence "Nevertheless, it is predictable that test results from different clinical laboratories using different NAAT platforms or even different techniques such as droplet digital PCR (ddPCR), which gives absolute count of viral copy number, will become increasingly comparable as secondary standards calibrated to WHO material and common international units (i.e., through conversion of Ct values to concentrations, e.g., log10 IU/mL) are adopted, as demonstrated in the study by Boan et al.".

Reviewer B

This is an important paper as the rationale is solid and the recommendations are valuable for public health assessment and reporting. My group has worked on SARS-CoV-2 respiratory infection for the past 3.5 years, building on work over 25 years with the Marsico Lung Institute at UNC and for over 10 years with folks in the School of Pharmacy at UNC studying viruses in mucosal barriers. Several points made in this review are "spot on", and consistent with our published work with many of my colleagues at UNC. Namely, viral load in the nasal passage and upper respiratory tract where clinical tests sample from, is extremely heterogeneous in infected individuals. We have shown using modeling that how rapidly a cell, from the moment of infection to the onset of shedding, as well as the shedding rate of infectious virions, are the absolute drivers of early infection. Further experimental tests following a high-titer nasal infection test would help medical diagnostics of who is at risk of spreading the infection to the deep lung. The more information we get out into the open literature the better off we will be, which is one of the main points of this review. Our papers noted above are listed here; the first and last ones are the most relevant to this review, as they reveal key mechanistic sources of extreme heterogeneity from a physiologically faithful respiratory infection model.

Modeling insights into SARS-CoV-2 respiratory tract infections prior to immune protection, A. Chen, T. Wessler, K. Daftari, K. Hinton, R. C. Boucher, R. Pickles, R. Freeman, S. K. Lai, M. G. Forest, Biophysical Journal 11742 https://doi.org/10.1016/j.bpj.2022.04.003 (2022)

Aerosol transport modeling: the key link between lung infections of individuals and populations, C. Darquenne, A. T. Borojeni, M. Colebank, M. G. Forest, B. Madas, M. Tawhai, Y. Jiang, Frontiers in Physiology 13:923945 DOI: 10.3389/fphys.2022.923945 (2022)

A hybrid discrete-continuum model of antibody and interferon immune responses to SARS-CoV-2 infection in the lung alveolar region, A. Aristotelous, A. Chen, M.G. Forest, J. Theoretical Biology 555, 111293 DOI: 10.1016/j.jtbi.2022.111293 (2022)

Antibody protection from SARS-CoV-2 respiratory tract exposure and infection, A. Chen, T. Wessler, M.G. Forest, J. Theoretical Biology 557 111334 DOI: 10.1016/j.jtbi.2022.111334 (2023) Modeling identifies variability in SARS-CoV-2 uptake and eclipse phase by infected cells as principal drivers of extreme variability in nasal viral load in the 48 hours post infection, J. Pearson, T. Wessler, A. Chen, R. Boucher, R. Freeman, S.K. Lai, R. Pickles, M.G. Forest, J. Theoretical Biology DOI 10.1016/j.jtbi.2023.111470 (2023)

- ANSWER: Thanks for these suggestions. We have included all these valuable comments and the relative two references as follows: "Importantly, viral load in the nose and upper respiratory tract, where clinical samples are collected, is highly heterogeneous in infected individuals. Interestingly, based on model studies (30,31), it has been shown that the time elapsed between cell infection and the onset of shedding, as well as the shedding rate of infectious virions, are absolutely critical for early infection. Therefore, further experimental tests following a nasal high titer infection test would help medical diagnostics to identify who is at enhanced risk of spreading the infection to the deep lung".

Reviewer C

Lippi and co-workers aimed to revise the role of viral load reporting in individuals infected with SARS-CoV-2 in mitigating the care in hospitals. The authors in particular focused on advantages more than the drawbacks of continuing to report the upper respiratory tract SARS-CoV-2 viral load in infected patients. General comments: This is an important topic to be revised with relevant clinical implications. However, I have some recommendations to improve the quality of the information and to make the review more homogeneous.

1. I would recommend the authors to discuss the limitations to the methods of using Ct values for viral load estimation. The authors should include a limitations section describing, how it is not uncommon for Ct values to vary significantly in the context of COVID-19 testing.

- ANSWER: This is very good point, which has been specifically addressed in replying to the other referee, as follows: "Importantly, the viral load in the nose and upper respiratory tract, where clinical samples are collected, is highly heterogeneous in infected individuals. Interestingly, based on model studies (30,31), it has been shown that the time elapsed between cell infection and the onset of shedding, as well as the shedding rate of infectious virions, are absolutely critical for early infection. Therefore, further experimental tests following a nasal high titer infection test would help medical diagnostics to identify who is at enhanced risk of spreading the infection to the deep lung". We have also added the following paragraph: "There are certainly some preanalytical, analytical, and postanalytical limitations to the measurement of SARS-CoV-2 viral load (i.e., primarily Ct) in upper respiratory tract specimens. In addition to preanalytical problems that may affect the collection and storage of swabs (different and/or

inappropriate sampling techniques, unsuitable media, and/or conditions for transport and storage), one of the most important drawbacks is the poor interlaboratory comparability, especially when different assays are used, mainly because the reference standard WHO 20/146 still seems to be underutilized. Inappropriate interpretation of Ct values during the clinical course of the disease may also bias the clinical reasoning.". Please consider that a full table is also available (Table 2), listing all possible drawbacks.

2. The factors affecting the Ct values, like variation between different methods, instrument variables, variable collection methods must be added in the limitations.

- ANSWER: Done, as follows (see above).

3. I recommend the authors to include and discuss these two important references in the text:

a. Magleby R, Westblade LF, Trzebucki A, Simon MS, Rajan M, Park J, Goyal P, Safford MM, Satlin MJ. Impact of Severe Acute Respiratory Syndrome Coronavirus 2 Viral Load on Risk of Intubation and Mortality Among Hospitalized Patients With Coronavirus Disease 2019. Clin Infect Dis. 2021 Dec 6;73(11):e4197-e4205. doi: 10.1093/cid/ciaa851. PMID: 32603425; PMCID: PMC7337625.

b. Tian D, Lin Z, Kriner EM, Esneault DJ, Tran J, DeVoto JC, Okami N, Greenberg RM, Yanofsky S, Ratnayaka S, Tran N, Livaccari M, Lampp ML, Wang N, Tim S, Norton P, Scott J, Hu TY, Garry R, Hamm L, Delafontaine P, Yin XM. Ct Values Do Not Predict Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) Transmissibility in College Students. J Mol Diagn. 2021 Sep;23(9):1078-1084. doi: 10.1016/j.jmoldx.2021.05.012. Epub 2021 Jun 5. PMID: 34102313; PMCID: PMC8178946.

- ANSWER: Done, a suggested (both references included): "However, further standardization processes would be needed to improve the clinical utility of reporting the Ct value. Although it is now highly likely that assessment of SARS-CoV-2 viral load would help identify individuals at higher risk for adverse outcome (32), its clinical relevance for identifying so-called super-spreaders is still debated in the current scientific literature, as demonstrated by Tian et al. (33), who showed that the individual viral load is not an efficient predictor of transmissibility."

4. The rest of the sections are more balanced and complete.

- ANSWER: Thanks. No changes needed.