Peer review

A good read and a worthwhile experiment. However, I have some concerns that need to be addressed.

You state “….In this study, I aimed to develop an alternative surveillance tool for the early detection of emerging SARS-CoV-2 variants and obtain individual data to provide comprehensive and individualized surveillance data to track the virus progression.”. Wastewater testing for Covid is already an established method to detect community spread. The PCR method does not necessarily detect genetic variants since it uses conserved sequences of the virus as templates. Hence, either remove these claims from the manuscript or substantiate them with references.

The last paragraph under “introduction” actually presents methods, results and conclusions. Please present relevant content under their appropriate sections in the manuscript.

Centrifugation parameters are reported as multiples of the number of times the force of gravity and a time. Please change RPM into “x g” for interoperability between labs.

please state the correct reporting semantics for “…..to bring each sample to the bottom of its tube…….”

You state “…..between 34 and 37 as compared to 31.3 with 200 copies of the virus in Standards” Is this the positive control? Include origin, and validation (certification). However, later in the manuscript, you mention a value of 20 copies “….The standard sample of 20 copies had a CT value of 34.6”. At still another place in the manuscript, you state that "…. (CT value around 31)…. was LOQ " Please recheck. How many replicates of sample and standard were performed? A literature search (see: https://doi.org/10.1128%2Fspectrum.04470-22 figure 1) shows that the CT (for various labs and instruments avg) for a PFU/mL of 20 corresponds with 28 as cycle threshold. Why is your value of 34.6 (or is it 31.3?) significantly greater? What was the standard deviation for this Ct value? Was the instrument set so that a Ct was reported when the fluorescence of the sample was more than 10? standard deviations of the positive fluorescence? Why was 20 PFU/mL chosen as the value for the positive control ? (I am assuming that the positive sample Ct was performed on the same or similar instrument and that mean and standard deviation of Ct is known as is the average fluorescence and its (the fluorescence’s standard deivation?) Were all these parameters able to be manually fed into the machine? If not, please describe in detail how the machine actually calculated the value of Ct from fluorescence (compared with positive control) and its internal algorithm if any. Please describe the procedure in sufficient detail and with sufficient justification so that it can be replicated in other labs.

At various places in the manuscript, you refer to samples as being a positive or a standard. Were these the same samples?

Why were the N1 and N2 primers not run simultaneously in the qPCR assay? Did your positive control not have both targets? By convention, both of these are run together.

You state “…..Thus, I reason that none of these environmental samples would be derived from an infectious virus”. I think what you mean is that none of these enivronmental samples would be capable of replication. Please check.

you state "….my results suggest that there are widespread trace amounts of RNA after the COVID-19 pandemic. " Please restate using the adjectives “non-replicable and non-infectious”

You state at multiple places in the manuscript "…..Additionally, using a CT value of 40 as a cutoff to diagnose COVID-19 in humans and animals may need revision." This needs references. The problem with qPCR is that it is a snapshot in time and does not necessarily reflect if the person will be positive in the future (initial stages of viral infection). There is also no correlation with culturability and CT or with infectivity and CT. Hence, please provide references for this suggestion or provide sufficient justification for why the Ct of 40 should be lowered.

For the Gel-electrophoresis, you have only presented 5 samples out of 12. Did all the samples show a band at the same mw at that of the positive control? I am assuming that the positive control contained the N2 gene product of the SARS at 20 copies RNA ?

Please rewrite the manuscript in 3rd person, past perfect tense. Please do not use non-scientific and subjective opinionated words or phrases such as "To my surprise, all environmenta……". Please check the entire manuscript and delete.

Was the internal control Ct within limits?

Did you do the majority of the work in the lab or was your work limited to collecting the samples?

Thanks to the reviewer for the constructive suggestions. I discussed with my mentor at the FDA and made changes to the manuscript. For a better view of the changes, I provided two documents, one with the track changes and the other the clean version.
Here are my responses (in blue) to the questions raised by the reviewer.
A good read and a worthwhile experiment. However, I have some concerns that need to be addressed.
You state "….In this study, I aimed to develop an alternative surveillance tool for the early detection of emerging SARS-CoV-2 variants and obtain individual data to provide comprehensive and individualized surveillance data to track the virus progression.". Wastewater testing for Covid is already an established method to detect community spread. The PCR method does not necessarily detect genetic variants since it uses conserved sequences of the virus as templates. Hence, either remove these claims from the manuscript or substantiate them with references.
Changed - Track changes have been made on Page 1 in the new version.
The last paragraph under "introduction" actually presents methods, results and conclusions. Please present relevant content under their appropriate sections in the manuscript.
Corrected - Track changes have been made on Page 3 in the new version.
Centrifugation parameters are reported as multiples of the number of times the force of gravity and a time. Please change RPM into "x g" for interoperability between labs.
Corrected - Track changes have been made on Page 4 in the new version.
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Yes, it is the positive control. The original record of the study states that 20 copies of the plasmid in the standard sample corresponded to Ct value of 34.6. The values mentioned in the manuscript are all correct.

How many replicates of sample and standard were performed? A literature search (see: https://doi.org/10.1128%2Fspectrum.04470-22 figure 1) shows that the CT (for various labs and instruments avg) for a PFU/mL of 20 corresponds with 28 as cycle threshold. Why is your value of 34.6 (or is it 31.3?) significantly greater? What was the standard deviation for this Ct value? Was the instrument set so that a Ct was reported when the fluorescence of the sample was more than 10? standard deviations of the positive fluorescence? Why was 20 PFU/mL chosen as the value for the positive control ? (I am assuming that the positive sample Ct was performed on the same or similar instrument and that mean and standard deviation of Ct is known as is the average fluorescence and its (the fluorescence's standard deivation?) Were all these parameters able to be manually fed into the machine? If not, please describe in detail how the machine actually calculated the value of Ct from fluorescence (compared with positive control) and its internal algorithm if any. Please describe the procedure in sufficient detail and with sufficient justification so that it can be replicated in other labs.

Regarding the comparison between the Ct values obtained in our results and those in published literature, "PFU/ml of 20" refers to the infectious titer of cultured viruses in the study referred to by the reviewer (https://doi.org/10.1128%2Fspectrum.04470-22 figure 1). However, the copies used in our standard curve refer to the calculated number of viral RNA copies based on a standard curve generated using 2019-nCoV_N_Positive Control (IDTDNA). These are two different standards and cannot be compared directly. In the samples used in the mentioned literature, one infectious viral particle (from the cultured virus) might contain multiple copies of virus genomic RNA and subgenomic RNA, including subgenomic RNA which encodes the N protein. In our standard sample there were exactly 200 copies of plasmids, which is why the Ct values in our assay were much lower than those reported in the referenced study.

All RT-PCR assays were done in duplicates on one instrument, with all parameters set with the same values across different assays. The standard deviations of each serially diluted standard with N1 and N2 primers/probe sets ranged from 0.007 to 2.489. These details have been added in Materials and Methods on Page 4 and 5.

At various places in the manuscript, you refer to samples as being a positive or a standard. Were these the same samples?

Yes, these were the same samples.

Why were the N1 and N2 primers not run simultaneously in the qPCR assay? Did your positive control not have both targets? By convention, both of these are run together.

As described in Table 1, N1 and N2 primers/probe sets cover different regions of the virus's sequence and were used in separate runs to confirm the results. The sequence of positive control does contain the region for both sets. If we used the two sets of primers/probe in one run, to our understanding, the RT-PCR would not distinguish the mixed PCR products (amplicons) and the results would not be interpretable. Hence, the two runs were done in two separate mixtures (not in the same tubes)

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You are correct - Track changes have been made on Page 6 in the new version.

you state "….my results suggest that there are widespread trace amounts of RNA after the COVID-19 pandemic. " Please restate using the adjectives "non-replicable and non-infectious"

Corrected and track changes have been made on Page 7 in the new version.

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The reference of cutoff Ct at 40 has been added to the manuscript (reference #18 on Page 3 and 7).
In terms of the correlation between Ct value and viral infection, there is a study on the relationship between the duration of illness and its Ct value. During the first week of COVID-19 infection, the Ct values varied between 26 and 28, which then increased to 32 on day 8 to 10. Severity of symptoms seemed to also correlate with Ct values (https://www.ncbi.nlm.nih.gov/pmc/articles/PMC10505265/). A sentence has been added on Page 7 and the reference #28 has been added.

For the Gel-electrophoresis, you have only presented 5 samples out of 12. Did all the samples show a band at the same mw at that of the positive control? I am assuming that the positive control contained the N2 gene product of the SARS at 20 copies RNA ?

All 12 samples were shown in the figure. The picture of gel electrophoresis contains two rows; the lanes are labelled from 1-6 on the top row and 7-12 on the bottom row. The positive control is labelled in red and the band is highlighted with a dotted rectangle. The molecular weight of each band is below 100 bp. Yes, the positive control contained the N2 primers/probe set and 20 copies of the plasmid.

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Track changes have been made on Pages 1, 5, 6, 7, and 8 in the new version.

Was the internal control Ct within limits?

Primers/probe set of RNase P (human ribonuclease P) was used as an internal control in the RT-PCR assay but the Ct values were “undetermined” in all the samples. This is most likely because the environmental samples contained no or undetectable human cells, which is unlike the human nasal swab that has human cells in the sample. For quality control, nuclease free water was used as template in the negative control and did not yield any amplicon even after 40 cycles. The commercial N positive control from IDNDNA was used as a template in the positive control and yielded an excellent standard curve.

Did you do the majority of the work in the lab or was your work limited to collecting the samples?

I performed all bench work pertaining to this study in addition to the field work. All sample processing, gel electrophoresis, RT-PCR, data analysis, etc. were done by me under the supervision of Dr. Wang and his postdoctoral trainees at the FDA.

Accepted