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Reviewer A

The authors have attempted to study the sensitivity efficacy of the RT-PCR test and aim to justify the possible pathways to validate the test results in a different way, which is indeed appreciable. However, the authors have to follow and implement the following comments.

1. The introduction should be refined on including the points on the importance of results and the significance of this study in regards to future research aids has to be explained in detail.

Response: Following the reviewer's suggestions, we now explain why pool testing plays a key role in the control of SARS-CoV-2 infections and provide specific examples of its potential use. Unfortunately, this has required re-writing most of the introduction. Most of the new content is in the first paragraph (lines 52-62) and the third paragraph (lines 73-80). Key ideas are: the relevance of widespread testing in the current context of the pandemic, the inequity of testing in low and high income country, and the role of pool testing in health care workers and the general population.

2. How this pooled sampling (positive samples mixing with negative sampling) will be helpful to understand the sensitivity of RT-PCR analysis for COVID-19 treating frontline workers? Please explain this in the introduction in detail. Looks very vague introduction.

Response: We appreciate your comment. We have addressed this issue by re-writing our introduction. Please see our response to the previous comment.

3. Why has a 1:89 ratio of positive and negative sampling has taken? (Line 81) Reason?

Response: This is just a misunderstanding. What we mean is that we tested at least 89 pools of 4, 8, 16, and 32 samples each. Table 1 shows the actual numbers of pools for pools of each size. We had 89 pools of 4 samples, 92 of 8 samples, 102 of 16 samples, and 105 of 32 samples. The number of pools is lower with lower pool size because sensitivity of the test increases and, therefore, a smaller number of pools is required to achieve a fixed precision.

4. What is the ratio strategy? Does each pool have different mixing numbers?

Response: We used pool of 32, 16, 8, and 4 samples because there was previous evidence to support these sizes and we wanted to identify what pool size would be

most efficient, depending on the prevalence of infections in a population. Even though we have pools of four different sizes (4, 8, 16, 32), each pool of 4 samples had 1 positive and three negative samples, each pool of 8 had 1 positive and 7 negative samples, and so on. Therefore, each pool of a given size had a fixed number of one positive sample and the rest of the samples (pool size minus 1) were negatives.

5. The results have shown the higher the pool is showing less sensitivity. Then what is the reason for mixing and performing this analysis?

Response: That is correct. The larger the pool size the lower the sensitivity of the pooled test. This makes sense, because on positive sample is diluted in a larger number of negative samples. For instance, if the same positive sample is included in a pool of size 4 and also in a pool of size 32, the concentration of viral particles would be lower in the pool of size 32. Therefore, the sensitivity for the pool of 32 would be lower than that of the pool of 4 samples. However, if we use pools of 32 instead of single tests, and the 32 individuals are negative, we could ascertain their status using only one test, instead of 32 tests. On the contrary, if there is only 1 positive in the pool of 32, and the pool is positive, we could split the pool in two pools of 16 and test these two pools, then we could split the pool of 16 that was positive in two pools of 8 and test these two pools, then we could split the pool of 8 that was positive in two pools of 4 and test these two pools, then we could do individual tests in each of the 4 samples from the pool of 4 that was positive. In total, we would use 1+2+2+2+4 = 11 tests, instead of 32 tests. However, unless the prevalence of current infection is very high, most of the pools would have no positive samples. In consequence, pool testing allows discarding infection in large numbers of people saving resources and time, and maximizes the number of people tested in the shortest time. Of course, we want to use a pool size with a high enough sensitivity, to avoid missing many positive cases. That is why we need to tests pools of different sizes. We conducted simulations in our study, based on the sensitivity of pool testing for different pool sizes, to evaluate the expected increase in testing efficiency.

6. 95% CI of each sample Ratio would be mentioned in the results section to give clear evidence of information.

Response: The ratio of positive to negative samples for pools of the same size is constant. For pools of size 4: 1/3; for pools of size 8: 1/7; for pool of size 16: 1/16; and for pools of 32: 1/31. Analyses were conducted separately for each pool size. Therefore, there was no need to estimate the variability of the positive to negative sample ratio for each pool size.

7. What is the use of low pooling sampling that will help time-consuming in this pandemic situation and how would you say this study will account for cost-effective and efficacy of Rt-PCR results?

Response: This is a very useful and appreciated suggestion. We have added the

following text to our discussion (lines 255-267):

It is also key for each lab to quantify the additional time required for preparing pools and for positive pools deconvolution to identify positive individuals. Additional time over single testing would depend on each lab specific requirements and resources, poll size used, and prevalence of active infection. This time should be taken into account while calculating the local cost-efficiency of pool testing. There is scarce data on pool testing as compare to individual testing, since this varies significant from lab to lab. However, findings from simulation studies in large populations suggest that pool testing for population-wide screening, such as in health care workers and essential personnel, could be 8-10 times faster than individual testing.[44, 45] Labs could also use matrix pool testing, i.e. 2-dimentional array of rows and columns, with each sample included in a row-pool and a column pool to avoid pool deconvolution.[46, 47] If both pools defining an intersecting cell in a 2-dimentional array were positive, then the shared sample in that cell would be positive, and no additional testing would be needed.[44] We have adopted this approach in our lab.

Please have a look and refine the answers. These questions are to be answered in the appropriate section

Reviewer B

The manuscript by Bautista et al describes the result of a pooling exercise for detecting SARS-CoV-2 by Rt-PCR. There are several important issues the authors should consider to improve their manuscript.

Methods

Line 80 page 4. It is unclear whether each positive sample was included in all 4 size pools or if it was a single positive sample in a given pool size.

Response: We have now clarified the issue by rewriting 97-99 as follows: Single positive specimens were mixed with 3, 7, 15, and 31 negative specimens to make pools of 4, 8, 16, and 32 samples each.

Lines 89-96. Most published reports on pool testing utilised mixed swab samples followed by extraction instead of pooling extracted samples which is technically more difficult, more time consuming and expensive. Authors should justify their approach particularly with regards to cost for developing countries. It this approach used simply because only extracted samples had been stored instead of swab collection buffers?

Response: The reviewer's appreciation is correct. We used this approach because our lab received samples collected from and only extracted samples had been stored instead of collection buffers. To clarify the issue, we have added the following in lines 99-100: *Pools of extracted RNA samples were used, instead of pre-extraction pools of nasopharyngeal swaps, because they had been stored and were readily available.*

Moreover, we have added the following statement to our discussion: *Another limitation of our study is the use of pools of mRNA, which were readily available, instead of pools of nasopharyngeal swaps before RNA extraction. The latter approach saves time and resources dedicated to RNA extraction in single samples. Therefore, our estimates of pool testing efficiency are lower than they would be if pools of nasopharyngeal swaps were used.*

Line 127. What is meant by 'nested pools'?

Response: It means pools made of samples from larger pools. For instance, if a pool of 32 samples were positive, the pool would be divided in two pools of 16 samples each for further testing. The two pools of 16 samples are nested within (are part of) the original pool of 32 samples.

Results.

There seem to be too little data analyses presented. If a given sample was included in each of the 4 pool formats (4, 8, 16 and 32) the reproducibility of Ct numbers for each pool size tested from undiluted samples above 34Ct 30-34Ct and <30Ct should be calculated. In each of these groups the actual number of samples resulting in Ct values above 40Ct (negative) should be indicated in each pool size.

Response: We feel we have conducted just the analyses needed to achieve our study objectives. However, it seems we neglected to mention an important detail that should address the reviewer's concern. After mRNA extraction, samples from an individual were stored in 1-4 aliquots. If aliquot A from positive patient 1234 was used in a pool, this sample was retested individually. Therefore, we had two "baseline" Ct values for patient 1234, one from when his original sample was tested and another one when his aliquot A was tested. If aliquot A was negative during the second test, when its pool was also tested, the pool was discarded, and a new pool was prepared, with a positive sample from a different patient. We estimated the change in Ct as the difference between the Ct from the pooled test and the original (first) test. This approach is the closest one to the ideal situation of preparing and testing a pool at the time when the positive sample included in the pool was first tested. However, we also conducted analysis comparing the Ct from the pool test to the Ct from the individual test in the positive sample, conducted when the pool was tested. Findings from this analysis were virtually identical to those from the analysis using the original (first) test of the positive sample. In consequence, the number of positive and negative samples in each pool was exactly as we planned.

To address the reviewer's concer, we have added the following text in Methods (lines XX, page YY):

Methods (lines 97-103): *Before including a originally positive sample in a pool, the sample was re-tested to confirm it was still positive and to get a new Ct value for that sample. The sample was included in the pool only if it was positive in this second individual test.*

Methods (lines 136-139): *We computed the average change in Ct value as the difference between the individual Ct of the positive sample in the pool, the first time the individual sample was testing, when received in our lab, and the Ct of its pool (Δ), and its standard deviation ($SD_Δ$).*

Methods (line 145-147): *The same analysis was also conducted using the Ct value from the positive sample in the pool, tested at the time when the pool including that sample was also tested.*

Results: *Findings from the analysis comparing the Ct from the test conducted in the positive sample when the sample was included in a pool and the Ct from the pool tests were virtually identically to those from the analysis using the original (first) Ct value for the positive sample.*

Discussion

Lines 177-78. Another way of predicting expected Ct changes is to use 1 log value difference corresponding to 3Ct change.

Response: We agree. However, the calculation applies to tests conducted in ideal circumstances (high reliability and a perfect log-linear relationship). We just report observed results, that are consistent with the formula indicated by the reviewer.

On the basis of their data, the authors should discuss the pool size they would recommend for use in areas where less than 1% positive rate is found. From the limited data presented, pools of 4 appear best suited for screening population in order to identify asymptomatic, infectious cases.

Response: We fully understand the reviewer's suggestion. However, which pool size should be used is highly dependent on context. Particularly, it is highly dependent on the prevalence of virus-shedding infected individuals in the population, which changes with the course of the epidemic. It also depends on what is the purpose of using pool testing. If the purpose were to make a diagnosis in high-risk population, like health care personnel, we would be inclined to use smaller pool sizes. We would also be inclined to use small pool size for the purpose of clinical diagnosis. We have used pools of size 8 and 16 in our university hospital. If the purpose were to conduct disease surveillance, where the main goal is excluding infecting individuals from the population, through quarantine and isolation of those positive, we would be inclined to use larger pool sizes. For those reasons, we feel it more appropriate to let the reader/use decide which is the best pool size for his/her particular population/ objective.