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Improvement of SARS-COV-2 screening using pooled sampling testing in limited RT-qPCR resources

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ABSTRACT

Background: COVID-19 is a worldwide pandemic representing the most challenging global health crisis currently. Screening tests availability are a problematic task due to resource-limited abilities of some countries using RT-qPCR technique for SARS-COV-2 detection.

Objective: To cope with these health emergencies, in particular with this COVID-19 pandemic, states with low molecular diagnostic resources must optimize their capacity in molecular tests. We aimed to design a simple and effective strategy to improve inputs in the RT-qPCR tests as we attempted to check the financial advisability of using such an approach by calculating reduction rate of the test unit cost.

Methods: The used RNA was taken from suspected Covid-19 positive people. Nasopharyngeal swabs were collected at Pasteur Institute Diagnostic Center, Constantine, Algeria, 2020. We have optimized a screening strategy by grouping 16 individuals per pool, without reducing the sensitivity of RT-qPCR.

Results: A 1/16 dilution of a positive sample was a practical limit that does not require the use of robotic systems or mathematical modeling to construct the pools. The financial analysis of our strategy has shown that the costs can be reduced to 90 %. The pooled testing strategy that was proven in this study could be recommended to help COVID-19 containment in countries with low potential screening infrastructures using RT-qPCR technique by reducing the number of tests required to identify all positive subjects.

1. Introduction

COVID-19 Pandemic is a global challenging health crisis, affecting social and economic sectors. It weakened entire nations, mainly low technological resources nations (Nkengasong and Mankoula, 2020; Schellekens and Sourrouille, 2020). Diagnostic tests using RT-qPCR technology have become the benchmark for SARS-CoV-2 diagnosis essential for pandemic control according to the World Health Organization. Diagnostic kits market witnesses a huge pressure at the worldwide level, particularly in developed countries due to the massive screening of their population. Nonetheless, this situation deprived many other countries to afford these tests for screening implementation, which is not necessarily massive, as is the case in Algeria. In addition to the difficulty of supplying KITs, there is another hindrance specific to RT-qPCR technology as it is not widely used in our country.

To cope with this situation and to bridge the weak number of the used tests, we implemented a pooled sample testing strategy aiming to increase the national capacities of COVID-19 screening by optimizing the low number of thermal-cyclers and RT-qPCR available Kits. In its simplest form, the pooling test works by constituting a set of individual samples, in the case where the pool is COVID-19 negative, all the individuals are identified as negative and if the pool is positive, further tests would be necessary to identify infected individuals in the group (Dorfman, 1943; Olivier Gossner, 2020). This approach has been

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successfully used in the countries with high technological potential to deal with the lack of screening means (Mutzel et al., 2020; Shani-Narkiss et al., 2020), as it has been proposed as a massive screening strategy (De Salazar et al., 2020; Bilder and Tebbs, 2012).

Nevertheless, the approaches proposed in the literature do not take into account the specific realities to countries with limited access to molecular diagnostic tools, namely RT-qPCR Kits and thermal cyclers. To that end, we tested in local conditions several actions in recently published group tests and tried to answer key questions about this approach:

What is the size of a pool so that a single positive individual remains detectable (Eis-Hübinger et al., 2020)? When should the samples be grouped, would it be appropriate to do so after or before the RNA extraction step (Powers, 2011)?

In the other hand, we aimed to check the financial advisability of using a such approach by calculating reduction rate of the test unit cost.

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2. Methods

The used RNA was taken from suspected Covid-19 positive people. Nasopharyngeal swabs were collected at Pasteur Institute Diagnostic Center, Constantine, Algeria, 2020. This study was approved by the “Ad-hoc ethical and review board committee of the national center of biotechnology research”. Given the deidentified nature of testing, individual patient consent was not required for this study.

At first, we tested RT-qPCR ability to detect a single positive sample diluted in a group of negative samples, without increasing the number of amplification cycles under standard conditions.

2.1. RNA extraction

All RNAs were extracted from nasopharyngeal samples; 200 μl of the universal transport media were used according to the conditions recommended by the manufacturer of the KIT (DAAN Gene Co., Ltd of Sun Yat-sen university China references of KIT DA0591).

2.2. Pooling

We used 12 RNAs extracted COVID-19 positive and 36 other RNAs from confirmed COVID-19 negative individuals. The COVID-19 status of all these samples has been previously and individually confirmed by RT-qPCR. Each of the 12 positive RNAs was grouped with negative RNAs according to different dilution factors (1/4, 1/8, 1/16 and 1/32). The RNAs were grouped at equal volumes (10 μl) in a 1,5 ml tube, then vortexed for 30 s and centrifuged at 800 g for 1 min.

2.3. RT-qPCR reaction

Was done under the conditions described by the KIT supplier, BGI biotechnologies (Wuhan Co Ltd China Catalog MFG030010). The detection threshold written on the used Kit, was of 100 copies / ml. RT-qPCR was performed on an ABI 7500 Applied Biosystems 7500 Real-Time PCR System device (ThermoFisher scientific USA) according to the program recommended by the KIT manufacturer. We used the same RT-qPCR conditions for the individual samples as for the pooled ones.

2.4. Ct values

Each obtained value in individual amplification was compared to that observed after the pooling. Ct manufacturer values were retained; the result was considered as positive if the value of the obtained Ct is ≤38. The machine background noise baseline (ABI7500-ThermoFisher scientific USA) was set as the individual samples obtained results and was not changed for the results analysis after pooling.

3. Results

3.1. Inclusivity

The observed Ct values following the amplification of the target ORF1ab-gene (KIT BGI) before or after pooling for each of the individuals are compared with each other and a statistical study was carried out to assess the performance of the detected RT-qPCR. Positive individuals were diluted to 1/8, 1/16, and 1/32 with negative Sars-Cov 2 ARN samples. All the Ct values after individual tests are below the positivity threshold recommended by the KIT manufacturer, namely <38. All the Ct values of the internal control of the amplification reaction (the β-actin gene) than either for individual or pooled samples were <31.

The Ct values in the 1/4 pool were one unit higher than those of the individual samples, while for the 1/8 and 1/16 dilution groups are 2–3 units higher, however, all the Ct values remain within the positivity threshold. In the group of 1/32, an increase in the observed Ct values was over 3 units making some samples negative 33 % (4/12).

3.2. The case of sample 2

At dilution ¼, the result displayed by the machine was not determined for the target gene and the internal control gene, therefore we suspect an internal concern with the reaction for this case.

3.3. Data performance analysis

In order to evaluate the performance of practical diluted samples for the massive PCR testing, statistical analyses have been achieved. The main test of this part was to find the stopping threshold of the diluted sample (Ct value). As described in method section; the mixture fraction started from dilution 1/4 to 1/32. We consider the obtained data as binary classification (Table 1).

For the samples in each dilution, we first assigned the number 1 to positive test observation; in this case, the observation is true positive, because each mixture contains one positive sample (Ct Value <38). Second, we assigned the number 0 to negative observation result, in this case the observation is false-negative (Ct Value >38), since the mixture contain a positive sample. The performance metrics can be generated by drawing values from a 2 × 2 contingency table; the confusion matrix in Table 2 summarizes the results of different fractions.

The performances of the results were all evaluated using the standard parameters for classification including Sensitivity and Precision (Fawcett, 2006; R Development Core Team, 2008).

| Code | Positive sample before dilution | Dilution |
|------|-----------------------------|----------|
|      |                             | 1/4 | 1/8 | 1/16 | 1/32 |
| 1    | 1                          | 1   | 1   | 1    | 1    |
| 2    | 1                          | ND  | 1   | 1    | 0    |
| 3    | 1                          | 1   | 1   | 1    | 0    |
| 4    | 1                          | 1   | 1   | 1    | 1    |
| 5    | 1                          | 1   | 1   | 1    | 1    |
| 6    | 1                          | 1   | 1   | 1    | 1    |
| 7    | 1                          | 1   | 1   | 1    | 1    |
| 8    | 1                          | 1   | 1   | 1    | 1    |
| 9    | 1                          | 1   | 1   | 1    | 1    |
| 10   | 1                          | 1   | 1   | 1    | 1    |
| 11   | 1                          | 1   | 1   | 1    | 0    |
| 12   | 1                          | 1   | 1   | 1    | 1    |

1 = positive, 0 = negative ND no determined.

Table 1: Results of tested mixtures before and after 1/4, 1/8, 1/16 and 1/32 dilutions.
Recall = Sensitivity = True Positive / True Positive + False Negative
Precision = Confidence = True Positive / True Positive + False Positive

Models with high sensitivity have fewer false-negatives, and models with high specificity have fewer false-positives (Positive test value = precision). Recall and precision can be reported by parameters that measure the combination of both, like F-measure, which is the harmonic mean of recall and precision:

\[ F = \frac{2 \times \text{Precision} \times \text{Recall}}{\text{Precision} + \text{Recall}} \]

The classification metrics give a good idea about the overall tests performance. In the diluted mixtures from 1/4 to 1/16, the three parameters were good, with high sensitivity values varying from 91.97% to 100% and F-measure 0.96. The advantage of PCR-based diagnostic is precision. In our study the precision values of all tests were perfect with a 100% value and were explained by the absence of false-positive observations for all tests. Concerning observation 2 in dilution 1/4, the test was not determined and the same result for internal control was displayed. This error was about 8.33%, but the real error, in which the false-negative error happens is equal to values divided by the total number of fraction 1/4 to 1/16, so it was equal to 2.77%. However, in 1/32 fraction dilution, the number of false-positive increased to 4 observations, by default, the sensitivity decreased to 66.67%, in conclusion we recommend a dilution limit threshold of 1/16 fraction dilution (Table 3).

We carried out a blind test of four (04) RNAs extracted from asymptomatic samples non-confirmed as Covid-19 positive. We diluted each of the samples in the 16th with RNAs from individuals tested negative for COVID-19. This test was carried out under the same conditions as above. The Ct values obtained for the four (04) tested samples did change the initial state of positivity. Nevertheless, an increase of 1–2 units of Ct values was observed in the pooled samples compared to those obtained individually.

### 3.4. Financial considerations

To determine the impact of financial considerations of this procedure; and whether the pooling method is a good procedure with important economic issues, we studied out the expected cost for financing this approach and evaluation of the total cost for the selected pool holding 16 patient samples. We noticed then a meaningful cost reduction comparing to individual test. We have proceeded with statistical language R (Van Domelen et al., 2018) and the "pool cost" function in the pooling package (De Winter, 2013), which enabled us to plot the relationship between total costs vs. pool size. Pooling works great in the two-sample t-test scenario (Shipitsyna et al., 2007), as it reduces the variance of each observation from \( \sigma^2 \) to \( \frac{\sigma^2}{g} \), where g is the pool size. To better illustrate the case, we supposed that we would study "costs vs. pool size" for \( d = 0.1775 \), where d is a numerical value designating the real modification in the group means, and the biomarker has a variance. We supposed that the global cost for 1000 tests RT-qPCR is about 15,000 in arbitrary values and the evaluation of total cost for pooling test is illustrated in Fig. 1.

### 4. Discussion

The growing demand for SARS-CoV-2 tests by RT-PCR has deprived some countries of access to a massive screening strategy, which has demanded a new approach so that the available laboratories can accommodate a large number of samples to analyze. Another limitation to our study was the low number of samples as well as the poor amount of literature as the virus appeared and mutated recently. Nucleic acid testing allows the use of sample pooling strategies, which have already been used in previous studies for a large number of pathogens (Mallapaty, 2020). Literature review on pooled test strategies in the case of COVID-19 discloses two main approaches: one purely mathematical (statistical), which seeks the precise estimation of the optimal size of the group beyond the search for the frequency of the positive target in this group (Bao et al., 2020).

This approach was made on a provisional basis and models situations that could be large and impossible to transpose to the specific features of certain countries. The other approach exposed methodologies with a large volume of samples, (using for example high-throughput automata) which could not be transposed to the reality of certain low-income countries. We believe that our results can help small laboratories meet the growing demand for SARS-CoV-2 tests and can be used as a full-scale experiment for their needs. The limited diagnostic test resources of the SARS-CoV-2 forced some laboratories to optimize their procedure to deal with the pandemic. We then proposed a simple and practical approach with groups of small numbers of individuals of 8 or 16 patients per test. The obtained results show that using RT-qPCR tests on pooled samples of 16 individuals, the reliability and reproducibility of the data (Ct values) is of 100%. The experimental proposed protocols in our

| Dilution | Precision | F-measure | Error | Recall | Position | Tested as |
|----------|-----------|-----------|-------|--------|----------|----------|
| 1/4      | 100.00 %  | 0.96      | 8.33 %| 91.67 %| Positive | Positive |
| 1/16     | 100.00 %  | 1        | 0.00 %| 100.00 %| Negative | Negative |
| 1/32     | 100.00 %  | 0.80      | 33.33 %| 66.67 %| Positive | Positive |

Fig. 1. Visualize Total Costs for Pooling Design as a Function of Pool Size. The figure shows a high-profile application of pooling method, in which the price is downscaled by reducing the number of assays by nearly 92%.
The pooled testing strategy that was proven in this study could be recommended to help COVID-19 containment in countries with low potential screening infrastructures using RT-qPCR technique by reducing the number of tests required to identify all positive subjects. Nonetheless further studies are required in order to agree and support our findings.

6. Limitations

The low number of samples as well as the poor amount of literature as the virus appeared and mutated recently.

7. Ethical approval

This study was approved by the “Ad’hoc ethical and review board committee of the national center of biotechnology research”. Given the deidentified nature of testing, individual patient consent was not required for this study.

8. Data availability

The data that support the findings of this study are available from the corresponding author upon request.

9. Author statement

All authors disclose any financial and personal relationships with other people or organizations that could inappropriately influence our work, as there is no interest to declare.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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