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Pooled-Testing for SARS-CoV-2 Reverse Transcription Polymerase Chain Reaction (RT-PCR) in asymptomatic healthcare workers in EL-Raghy isolation COVID-19 hospital, Assiut University

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Abstract

COVID-19 pandemic is a substantial challenge for healthcare systems. Severe acute respiratory syndrome coronavirus (SARS-CoV-2) reverse transcription-polymerase chain reaction (RT-PCR) tests are considered the gold standard technique for diagnosis of symptomatic and asymptomatic infectious viral carriers and for screening special or at-risk populations. The pooled testing procedure is commonly used to reduce the cost of screening a large number of individuals for infectious diseases. This work was conducted to verify the accuracy of the standard SARS COV-2 RT- real-time PCR kit for detecting a single positive sample in a pool of negative samples. Kit verification using negative and positive samples was performed for the selection of the target pool sizes. RNA extracts from 443 healthcare workers, after 15 days' rotation in EL-Raghy Isolation COVID-19 Hospital, Assiut University during the first outbreak of COVID-19 pandemic (the period from June to September 2020) were obtained and tested. Sixty-three different pool sizes (2, 3, 4, 5, 6, 7, 8, 9, and 10) were tested for the presence of SARS-CoV-2 using RT-qPCR. Of these, 53 pools (84.1%) were negatives and 10 pools (15.9 %) tested positive. The individual number of SARS- COV 2 RT-PCR tests used in different pool sizes was 40 tests. The total number of SARS- COV 2 RT-PCR test used in this study was 110 tests instead of 443 tests which reflect a decrease in cost up to 75.16%. In conclusion, the suggested pooling strategy can reduce testing loads which enable substantial savings in reagent costs, technical burden, and time to generate laboratory results.

Keywords: COVID-19, Pooled testing, RT-PCR.

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Introduction

Coronaviruses are large, enveloped RNA-positive-stranded viruses responsible for infecting mammalian and avian species. In December 2019, a new coronavirus (CoV) emerged in China to cause an acute respiratory

disease known as coronavirus disease 19 (COVID-19). It was first recognized in Wuhan, Hubei province, China. The virus was identified to be a beta-corona virus related to severe acute respiratory syndrome coronavirus (SARS-CoV).[1] New Covid-19 virus is the third known coronavirus to cross the species barrier and cause severe respiratory infections in humans following SARS-CoV in 2003 and the Middle East respiratory syndrome in 2012.[2]

During 2020, the number of confirmed COVID-19 cases was constantly increasing worldwide. Considering the Asian and European regions, a steep increase in cases was observed in low-income countries by the end of March 2020.[3] It is problematic to quantify the exact size of this pandemic as it would be necessary to count all cases including not only severe and symptomatic cases but also mild ones. Unfortunately, to date, there is no global and standard response to the pandemic, and each country is facing the crisis based on its health facilities, expertise, and hypotheses. Thus, there are different criteria for testing, hospitalization, and estimating of cases making it difficult to calculate the number of people affected by the epidemic.[4] Tracking the spread of COVID-19 is essential to mounting an effective public health response, understanding the current impact of the virus, and ensuring that health systems are prepared. The most accurate way of identifying COVID-19 is through testing samples using the reverse transcription-polymerase chain reaction or RT-PCR.[5, 6] However, facilities for RT-PCR are limited, even in high-income countries, and further, test costs are high both in terms of consumables and trained technicians.[7] Delays in testing have slowed the public health response in Europe and the United States, and challenges to testing in low- and middle-income countries are even greater.[8]

In the absence of sufficient RT-PCR capacity, an alternative approach that may provide this essential data in a way that is course-grained but at lower cost, is by using pooling of samples from multiple patients and performing RT-PCR on this combined sample. Pooling and group testing can be very effective in reducing the number of tests required to both identify infected people in a population and to estimate the infection rate in a population.[9] This work aimed to assess the accuracy of the standard SARS COV-2 real-time PCR kit for detecting a single positive sample in a pool of negative samples.

Materials and Methods

This study included 443 Healthcare workers after 15 days' rotation in EL-Raghy Isolation COVID-19 Hospital, Assiut University during the first outbreak of the COVID-19 pandemic (the period from June to August 2020). The study protocol was reviewed and approved by the Research Ethical Committee of the Molecular Biology Research & Studies Institute (MBRSI, dated August 2021). The practical part of this study was carried out in the Immunology Unit & Molecular Biology Laboratory, Clinical Pathology Department, Assiut University Hospital.

RNA extraction was performed using, nasopharyngeal and oropharyngeal swabs (Viral transport medium tube with Dacron swabs, Wellkang Ltd, England, UK) were used. Sample collection was performed according to CDC.[10] RNA isolation and purification were performed by QIAamp Viral RNA Mini Kit (QIAGEN: lot Number 52906, Germany) based on the binding of RNA to the silica membrane in fast spin-column (QIAcube Connect Automatic Nucleic Acid Extractor QIAGEN, USA). Assessment of SARS-CoV-2 viral RNA was performed by an RT-PCR COVID-19 assay (genesig® Real-Time PCR assay supplied by Primer Design, UK, SO53 4DG).

For the screening of SARS-CoV-2 in asymptomatic healthcare, we set up a pooling system of nasopharyngeal samples. A preliminary Verification Experiment was used for evaluation and to make sure that this procedure did not imply a significant loss of diagnostic sensitivity. The Verification Experiment was performed using a total of 150 leftover specimens that had tested negative with cycle threshold (Ct.) value of 39, and 25 tested positive specimens (five samples at the different log with Ct. ranged from 38, 35±1, 32±1, 29±1, and 26±1). All samples included in the positive and negative pools were tested individually. Different pool sizes (pool of five,

ten, twenty, thirty, forty, and fifty) were performed (Table 1). RNA extraction was performed from each pool size (total number 30 tubes, Table 1) and RT- PCR for SARS-CoV-2 was performed for each pool.

Results

Results of the Verification Experiment

Table 1 and Figures 1, 2 and 3 show the results of the Verification Experiment. At a Ct. value of 26±1 (high positive samples), SARS-COV 2 RT-PCR showed positive results in all pool sizes (from pool of 5 up to pool of 50) with shift in Ct. value from 26±1 up to 30.69. At Ct. value of 32±1 (medium positive samples) SARS-COV 2 RT- PCR showed positive results in pool size (from pool of 5 up to pool of 40, but not detected in pool of 50) with a shift in Ct. value from 32±1 up to 37.8. While at a Ct. value of 38 (weak positive), SARS-COV2 RT- PCR showed positive results in pool size of 5 and 10, but not detected in other pool sizes (pool of 20,30,40 and 50) with shift in Ct. value from 38 up to 39.85.

Table 1. Cutoff (Ct.) values of SARS-COV-2 RT-PCR of the Verification Experiment.

Ct. value	Pool Size					
	5 Samples	10 Samples	20 Samples	30 Samples	40 Samples	50 Samples
38	39.8	39.85	-	-	-	-
35±1	36.9	38.6	39.6	44.5	-	-
32±1	34.6	35.4	36.09	37.0	37.8	-
29±1	31.5	32.5	33.07	33.44	33.8	34.3
26±1	27.9	28.4	29.73	30.24	30.09	30.69

The different pool sizes were prepared as follow:
Pool of five: one PCR positive sample from each cutoff level plus 4 randomly selected PCR negative samples,
Pool of Ten: one PCR positive sample from each cutoff level plus 9 randomly selected PCR negative samples,
Pool of twenty: one PCR positive sample from each cutoff level plus 19 randomly selected PCR negative samples,
Pool of Thirty: one PCR positive sample from each cutoff level plus 29 randomly selected PCR negative samples,
Pool of forty: one PCR positive sample from each cutoff level plus 39 randomly selected PCR negative samples,
Pool of fifty: one PCR positive sample from each cutoff level plus 49 randomly selected PCR negative samples.

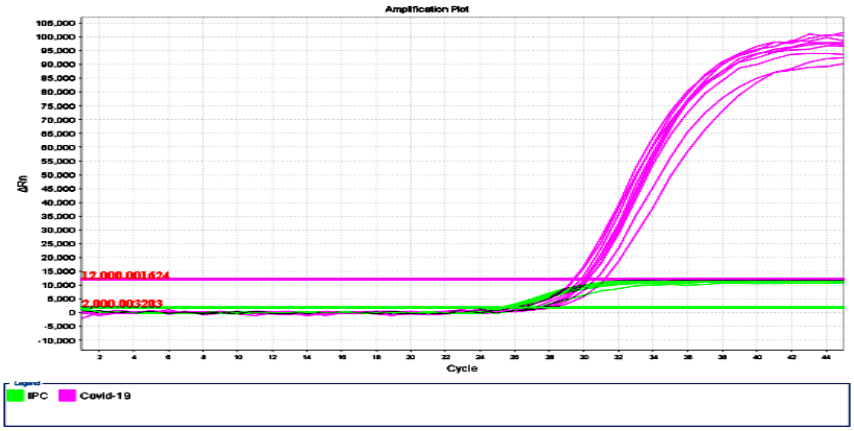

Figure 1. SARS-COV-2 RT-PCR using Ct. equal to 26±1 in different pool sizes**.**

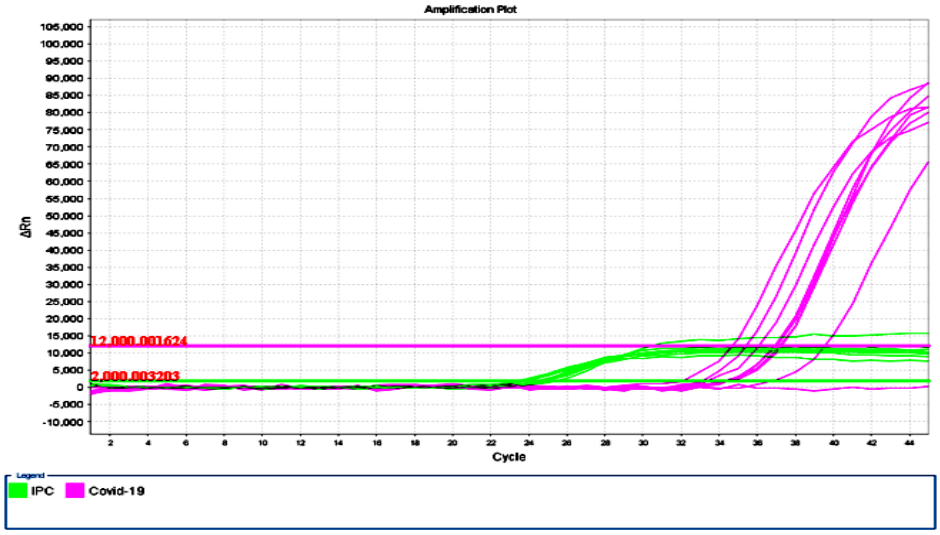

Figure 2. SARS-COV-2 RT-PCR using Ct. equal to 32±1 in different pool sizes**.**

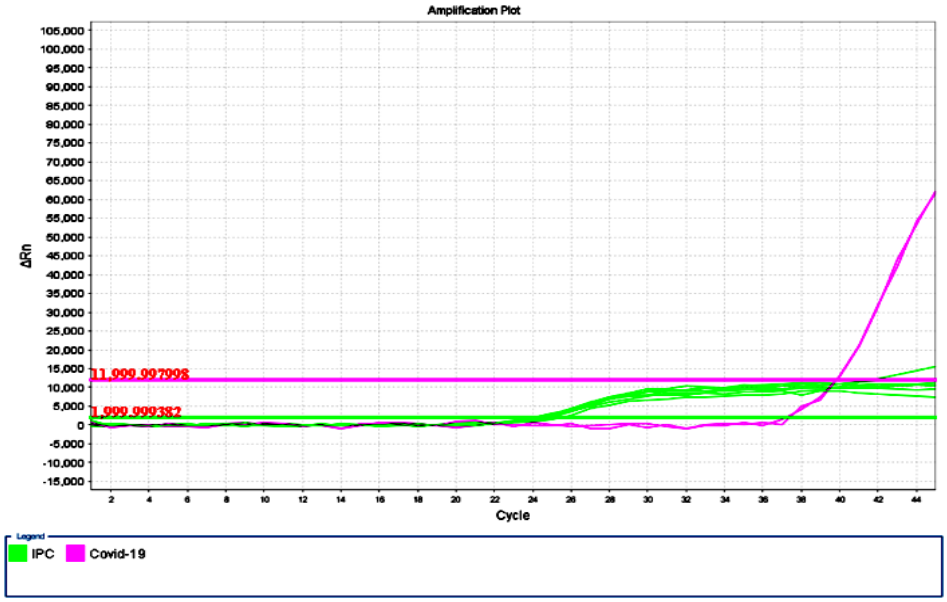

Figure 3. SARS - COV-2 RT-PCR using Ct. equal to 38 in different pool sizes**.**

According to the results of the Verification Experiment, we selected pool size of ten or less for examining of samples collected from healthcare workers and increased the Ct. value of negative to a Ct. of 40. This part of the study included samples from 443 healthcare workers. Samples were assembled in 63 pools, with different pool sizes, according to their collection during shifts in EL-Raghy Isolation COVID-19 Hospital, Assiut University during the first outbreak of COVID-19 pandemic. Table 2 shows their demographic data including age, sex, job categories and number of pools in the studied group. Of the 63 examined pools, 53 pools (84.1%) were negative and 10 positive pools (15.9 %). The number of the negative pools was significantly higher than the positive pools (*P*=0.001). Table 3 shows the different pool sizes among the studied group according to the work shift in EL-Raghy Isolation COVID-19 Hospital, Assiut University during the first outbreak of the COVID-19 pandemic. Table 4 shows the pool size ranged from a pool of ten samples (n=21, 33.3%) and a pool of two samples (n=4, 6.3%).

The SARS-COV 2 RT-PCR positive pools were then tested individually in a total of 40 tests. Of these 16 healthcare workers tested positive. The total number of SARS-COV 2 RT-PCR tests, in pools as used in this study, was 110 tests instead of 443 individual tests which reflects a significant decrease in the cost tests by 75.16%.

Table 2. Demographic data of the 443 healthcare workers.

Item	No (%)	*P* value
Age (years)		
Range	21- 45	
mean± SD	29±4.3	
Sex		
Male	159 (35.9)	
Female	284 (64.1)	
Job categories		
Physician	130 (29.3)**	
Nurse	251 (56.6)*#&	*P* = 0.001**
Health workers	54 (12.1)	*P* = 0.01*, *P* = 0.001#&
Other job categories	8 (1.8)	
Total pool number	63	
Negative pools	53 (84.1)	
Positive pools	10 (15.9)	

P value of: **Physician vs other job categories, *Nurse versus physician doctors, #Nurse versus workers, & Nurse versus other job categories. *P* <0.05 is significant.

Table 3. Number of negative and positive pools per month in the studied group during the first COVID 19 outbreak.

Month of sample collection	Negative pools No (%)	Positive pools No (%)	Total pools tested
June	17 (73.9)	6 (26.1)	23
July	16 (84.2)	3 (15.8)	19
August	11 (100)	0 (0)	11
September	9 (90)	1 (10)	10
Total pools	53	10	63

Table 4. Different pool size among the studied group

Pool size	Number of pools (63)	%
Pool of 10	21	33.3%
Pool of 9	4	6.3%
Pool of 8	7	11.1%
Pool of 7	3	4.8%
Pool of 6	8	12.6%
Pool of 5	4	6.3%
Pool of 4	8	12.6%
Pool of 3	4	6.3%
Pool of 2	4	6.3%

Discussion

For evolving and wide spreading outbreaks of infectious diseases like COVID-19, extensive individual tests are not only costly but also time-consuming. It has become important to come up with new ways to conserve the reagents used for diagnostic tests. At the same time, as the disease is novel, it is of value to validate any modifications to the testing process before universal adoption. Pooling diagnostic tests have been applied in other infectious diseases and are especially attractive as it requires no additional training, equipment, or materials.[12,13]

The present study aimed to validate pool testing procedures for detection of SARS COV-2 RNA by RT-PCR in naso and oropharyngeal swabs of healthcare worker (physicians, nurses and health workers) after 15 days of isolation in El-Raghy isolation hospital. This concept is in consistent with Bilder et al., 2009[14] who reported that the informative procedures rely on the basic idea that individuals have different risks of being positive and the retest of individuals based on the largest probabilities instead of selecting individuals randomly. Bilder et al., 2010,[15] also reported that informative retesting can reduce testing loads further when compared to their corresponding non-informative.

In the current study, results of the Verification Experiment revealed that the selected pool sizes were pool of ten or less, since we found that a single clinical sample with SARS-CoV-2 RNA can be consistently detected in a pool of 5 and 10 samples at a Ct. of 38 (weak positive) with shift in Ct. up to 39.8±0.03. For high-fold pooling (from pool of 20 up to pool of 50) our data shows an estimated false negative rate between Ct. 32±1 and Ct. 35±1. These results are supported by data of a study by Yelin et al., 2020[13] who found that a single clinical sample with SARS-CoV-2 RNA can be consistently detected in a pool of 16-32 samples. They also found that pooling in this way may lead to linear increase in the Ct. and the sensitivity reached to 96% for a pool size of 16 samples, and high-fold pooling, showed an estimated false negative rate. Moreover, they also found that these results can be used not only for pooling, but also in multiplexing and any other signal compression techniques where samples are mixed to reduce the number of tests and to develop mathematical and computational tools tailored for the pooling of SARS-CoV-2 tests.

Yelin et al., 2020[16] and Herper et al., 2020[17] also found that the optimization for the detection of low-concentration RNA and the detection of samples with even lower signals may warrant the use of smaller pools. Alternatively, adding a few additional PCR cycles could be considered as a means to increase detection rate of such low viral load samples. In general, as RT-qPCR kits and protocols vary internationally, use of suggested pooling may require validation for each specific setting. Consequently, in this study we performed a Verification Experiment using different pool sizes and different Ct. values including Ct. 38 which represent the low positive value.

In the present study the incidence of SARS COV 2 positive pools by RT PCR on nasopharyngeal swaps among the healthcare workers was 15.8%. This finding is in consistent with those of Yelin et al., 2020[16] who reported that pooling technique is especially useful for routine community survey and for monitoring of cohesive groups. Local and global epidemic response critically depends on determining carriage frequency in the population, which is greatly enabled by pooling techniques. Furthermore, pooling techniques can be used for routine monitoring of essential work groups, such as hospital staff, military units, and factory workers. While the frequency of infection in these groups may be low, diagnosing even a single positive person typically requires quarantine of the entire group to prevent further spread in the community. In these surveillance applications, pooling may allow more routine monitoring and detection of low frequency of carriage thereby informing policy makers, reducing transmission, and alleviating the strain on healthcare services.

The prevalence of SARS COV 2 positive cases among the studied population was 16 healthcare workers out of 443 (3.6%) and the numbers of tests used in 63 pools were 110

(24.8%). This finding is in consistent with those of Wacharapluesadee et al., 2020[18], who reported that the specimen pooling would certainly reduce the cost. For example, if 1% of the population is infected, pooling 10 specimens can reduce the cost of laboratory operation by about 80%. However, in the case of 10% prevalence, specimen pooling will only save 24.87%, as positive pooled samples will need to be individually tested. Therefore, pooling samples is especially useful in areas with low prevalence rates, or when conducting proactive surveillance in areas of low infection rate. Proactive surveillance, particularly in asymptomatic cases, remains a challenge to surmount in order to exit lockdown, as screening on a large scale is required.

Moreover, Hogan et al., 2020[19] also reported that specimen pooling is a method of screening large number of patients for an infection, and typically involves combining multiple patient specimens into a single test sample, then testing multiple such samples. This approach has the advantage of cost-effectiveness and speed and was used to retrospectively screen for COVID-19 in specimens that were negative for common respiratory viruses earlier in the course of the pandemic in the United States.

In the present study the number of positive pools increased in June (60%) and July (30%) comparing to August and September. Such findings were explained by Bullard et al., 2020[20] who reported that the adaptive pooling approaches can maximize resource saving under a fluctuating prevalence rate. The fraction of positive samples tested in pools can vary over time due to multiple factors affecting the epidemic kinetics, including changes in public health mitigation measures (for example, social distancing regulations, travel restriction, lockdown, and school closure)

Moreover, Barak et al., 2021[21] reported that the rising in infection rates have limited the pooling strategy usefulness. Because pooled samples must be broken apart and tested separately in the case of a positive result, pooled testing is inefficient in populations with high positivity rates. They also noted that the cutoff for positivity rates varied with the number of samples a laboratory pooled together. For instance, at a positivity rate of 10% it would make no sense to run pools of 10 samples, but it might still make sense to run pools of three or four samples.

According to the samples referred from El-Raghy Assiut University Isolation Hospital, pool of 10 represented 33.3% of referred pools followed by pool of 6 and 4 then pool of 5, 3, 2 and finally pool of 7. The higher frequency of positive pool was reported in pool of 10, follow by pool of 6 then pool of 9 then pool of 3 and pool of 2. No significant difference was observed between different pool sizes. These findings are supported by those of Wacharapluesadee et al., 2020[18] who found that the specimen pooling did not lower the sensitivity of PCR testing but actually increased the viral concentration when more than one positive sample was present in the same pool, i.e., combining the viral amount from 2 samples in the same extraction tube.

In conclusion, the pooling approach can reduce RT-PCR test loads, enable substantial savings in reagent costs and savings technical burden and time to generate laboratory results.

Author Contributions

EHM, AME designed the idea and the tools of the study. AME, MFA, DTK performed the laboratory work. RME made the statistical analysis. DTK examined the patients. MFA, DTK collected samples. All authors participated in writing and reviewing the paper.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Ethical approval

The study protocol was reviewed and approved by the Research Ethical Committee of the Molecular Biology Research & Studies Institute (MBRSI, dated August 2021).

Informed consent

A signed consent form was obtained from each study participant.

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