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SHORT COMMUNICATION

Prospective evaluation of specimen pooling strategy for detection of SARS-CoV-2 using pools of five and six specimens

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Abstract The increased demand for SARS-CoV-2 molecular testing during the COVID-19 pandemic resulted in shortage of reagents and consumables. Pooling of specimens could be an alternative strategy to overcome these problems. Initial evaluation of the pooling strategy was performed using known positive specimens, previously tested individually, and their respective pools of plus four (5X), five (6X) and nine (10X) known negative specimens. Subsequently, 35 positive 5X and 35 positive 6X pools containing only one positive specimen per pool were analyzed prospectively regarding the difference in Ct values in pooled versus individual specimens. When the number of samples in the pool were five or six, the average deviation of Ct differences was < 1; therefore, this strategy was followed in the prospective study. Significant difference in Ct values was observed in positive specimens when tested individually and in 5X pools ($p = 0.006$), while the difference was not significant when positive specimens were tested individually and in 6X pools ($p = 0.07$). The difference in Ct values was not significant between the 5X and 6X pools. Testing in pools of five or six specimens is a reliable option for SARS-CoV-2 RNA detection when mass testing is needed.

Keywords SARS-CoV-2 · COVID-19 · Pooling · Molecular test · Real-time RT-PCR

The ongoing COVID-19 pandemic caused by SARS-CoV-2, has resulted in an unprecedented public-health crisis of global dimensions with millions of confirmed cases and thousands of deaths reported to the World Health Organization (WHO 2020. Novel Coronavirus (COVID-19), https://covid19.who.int/). To control the spread of the disease by setting appropriate quarantine measures, massive and rapid diagnosis is becoming mandatory in order to indentify the infected persons. However, the high demand for SARS-CoV-2 molecular testing during the pandemic resulted into increased work load in the diagnostic laboratories and shortage of reagents and consumables. Testing patient specimens in pools is considered appropriate strategy to overcome these difficulties [3]. If the pool test results negative, all samples are considered negative, while the samples should be tested individually when the result of the pool is positive [12]. Recent studies showed the efficiency of pool sample testing during the pandemic [3–9]; Abdalhamid et al., supported that the pool size of five samples should be considered as the best choice [1]. In the present study we evaluated the pooling strategy using five (5X), six (6X) and ten (10X) specimens per pool. Since better results were taken in the 5X and 6X pools, we followed this strategy prospectively by testing nasopahryngeal specimens, and we compared the cycle threshold (Ct) differences of positive samples when tested individually and in pools.

In order to check the Ct differences between SARS-CoV-2 positive samples tested individually and in 5X, 6X and 10X pools, 5 known SARS-CoV-2 positive nasopahryngeal samples with Ct values ranging from 26.63 to 36.52 (mean 31.66), and 9 SARS-CoV-2 negative nasopahryngeal samples were used for an initial experiment. Viral RNA was extracted using the KingFisher Flex platform (ThermoFisher Scientific) which has a capacity for 200–400 µL initial volume. The volumes per individual

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Table 1 Volume per specimen and final volume used for 5X, 6X and 10X pooling

Pool size	Volume (μL) per specimen	Final volume (μL)
5X	70	350
6X	65	390
10X	40	400

specimen and the final volumes per reaction are shown in Table 1.

The RNAs were subjected to real time reverse transcription-polymerase chain reaction (RT-PCR) amplification applying the protocol described by the Institute Pasteur (IP) and distributed through WHO. The sensitivity of the assay in terms of 95% hit rate is about 100 copies of RNA genome equivalent per reaction, while no reactivity was seen when tested with specimens known to be positive for a panel of respiratory viruses (real-time-rt-pcr-assays-for-the-detection-of-sars-cov-2-institut-pasteur-paris.pdf).

Since best results were taken using 5X and 6X pools, we followed this procedure for the prospective study which included 70 (35 5X and 35 6X) PCR-positive pools of nasopharyngeal specimens. They corresponded to a total number of 385 single samples (175 samples in 5X pools, and 210 samples in 6X pools). Only pools containing one positive specimen per pool were included in the analysis. RNA extraction and RT-PCR were performed in the pooled and individual samples following the above-mentioned protocol.

Descriptive statistics were used to calculate the mean, minimum, maximum and standard deviation of the Ct values corresponding to the positive specimens when tested individually and in pools. The differences of the Ct values between pooled and individually tested specimens were calculated. Comparison of these differences and comparison of the differences between 5 and 6X pools were performed. The Mann–Whitney U test was used for the comparison of the mean values (criterion of significance $p < 0.05$). Statistical analyses were performed using IBM SPSS Statistics 21. Ct values were considered low, moderate or high when they were < 25, 25–30 and > 30, respectively.

All five known positive samples, when tested in 5X, 6X and 10X pools, resulted positive. The Ct differences between samples tested individually and in 5X pools ranged from −0.45 to 1.08 (mean 0.28), between samples tested individually and in 6X pools ranged from −1.53 to 1.40 (mean 0.33) and between samples tested individually and in 10X pools ranged from −0.39 to 3.42 (mean 1.9) (Table 2). The average deviation of Ct differences was < 1 only in the 5X and 6X pools; therefore, this strategy was followed in the prospective study.

The Ct values of the specimens in the 5X and 6X pools, together with those of the respective positive individual samples, and the related Ct differences are shown in Table 3a and b, respectively. The mean Ct value of the positive specimens in the 5X pools was 28.57 [range 19.43–38.80, standard deviation (SD) 5.16], while, the respective value was 26.21 (range 13.11–36.99, SD 5.05) when tested individually ($p = 0.006$). The mean Ct value of the positive specimens in the 6X pools was 30.00 (range 19.44–38.42, SD 5.21), while the respective value was 28.31 (range 16.24–38.47, SD 5.33) when they were tested individually ($p = 0.07$). The Mann–Whitney U test did not show significance between the differences of the two groups ($p = 0.293$), suggesting that testing in pools of 5X or 6X is equally acceptable.

Testing samples in pools is a strategy that has been successfully applied for years for various pathogens. Currently, this strategy is considered even more reliable because of the development of highly sensitive molecular assays. The increased demand for SARS-CoV-2 testing

Table 2 Cycle threshold values of known SARS-CoV-2 positive samples tested individually and in pools. Pool-individual Ct difference 5X range: −0.45–1.08. mean: 0.28; pool-individual Ct difference 6X range: −1.53–1.40. mean: 0.33; pool-individual Ct difference 10X range: −0.39–3.42. mean: 1.9. AVEDEV: average of the absolute deviations

Cycle threshold							
Specimen	Single	Pool 5X	Pool-individual difference	Pool 6X	Pool-individual difference	Pool 10X	Pool-individual difference
A	26.63	27.71	1.08	27.21	0.58	29.04	2.41
B	30.23	31.18	0.95	31.03	0.80	31.30	1.07
C	31.92	31.98	0.06	32.35	0.43	31.53	−0.39
D	33.02	32.57	−0.45	31.49	-1.53	36.01	2.99
E	36.52	36.28	−0.24	37.92	1.40	39.94	3.42
MEAN	31.66	31.94	0.28	32.00	0.33	33.56	1.9
AVEDEV			0.58		0.74		1.24

Table 3 Differences at cycle threshold (Ct) values of specimens tested in 5X pools vs individually tested (single) ($p = 0.006$), and specimens tested in 6X pools vs individually tested ($p = 0.07$), in increasing order of Cts. Comparison between differences (1 and 2) was not significant ($p = 0.293$). SD: standard deviation, AVEDEV: average of the absolute deviations

Cycle threshold						
	5X pool	Single	Difference (1)	6X pool	Single	Difference (2)
	19.43	19.26	0.17	22.54	19.31	3.23
	19.76	17.02	2.74	23.00	20.33	3.33
	19.91	13.11	6.80	23.15	19.26	3.89
	20.77	20.56	0.21	23.25	21.43	1.82
	22.55	20.37	2.18	23.68	22.85	0.83
	24.14	21.67	2.47	24.73	26.53	-1.80
	24.38	21.48	2.90	25.77	20.73	5.04
	25.75	31.92	-6.17	26.07	27.84	-1.77
	26.78	22.25	4.53	26.38	22.58	3.80
	28.42	23.20	5.22	27.10	24.18	2.92
	29.10	27.36	1.74	27.75	24.17	3.58
	29.54	27.58	1.96	28.21	27.08	1.13
	29.66	22.18	7.48	28.98	27.73	1.25
	29.76	29.57	0.19	29.95	26.34	3.61
	29.80	25.22	4.58	30.26	30.59	-0.33
	29.81	22.50	7.31	30.48	25.87	4.61
	29.89	26.41	3.48	30.75	28.18	2.57
	29.90	29.15	0.75	31.40	28.62	2.78
	30.26	24.02	6.24	31.53	25.50	6.03
	31.21	28.89	2.32	32.26	31.77	0.49
	31.37	26.11	5.26	33.10	32.50	0.60
	31.54	30.47	1.07	33.14	30.41	2.73
	31.61	30.22	1.39	33.45	27.85	5.60
	31.80	29.30	2.50	33.57	33.04	0.53
	31.81	29.46	2.35	33.67	33.26	0.41
	32.36	27.50	4.86	34.32	33.74	0.58
	32.50	28.48	4.02	34.38	33.95	0.43
	32.61	30.06	2.55	34.81	31.10	3.71
	33.11	31.41	1.70	34.82	33.68	1.14
	33.66	31.24	2.42	36.15	30.24	5.91
	33.68	31.82	1.86	36.58	32.59	3.99
	34.95	30.12	4.83	37.47	34.43	3.04
	35.61	36.99	-1.38	38.11	36.31	1.80
	35.91	28.62	7.29	38.26	38.47	-0.21
	38.80	31.34	7.46	38.42	33.24	5.18
MEAN	28.57	26.21		30.00	28.31	
SD	5.16	5.05		5.21	5.33	
AVEDEV			2.13			1.76

during the COVID-19 pandemic has led to an almost mandatory implementation of this procedure. However, it has to be mentioned that pooling is beneficial when the prevalence of the disease is low (< 4%); otherwise, the number of positive pools which should be re-tested seperately would be high.

The major concern of the pooling strategy is to avoid false negative results. A recent predictive algorithm indicated that a pooling ratio of 1 to 5 is expected to retain accuracy [1]. Sufficient diagnostic accuracy has been reported even when 30 samples of asymptomatic people were tested in the same pool [6]. In the current study was shown that the Ct difference of the positive specimens were not significantly affected when passing from 5 to 6X pool size. These results enhance previously published data indicating that pooling did not affect the sensitivity of SARS-CoV-2 detection when the Ct of the original specimen was lower than 35 [11]. It was of interest that positive

samples were detected in the 5X and 6X pools even when the Ct value was > 37 (which is usually used as cut-off). However, it cannot be excluded that positive samples with high Ct might be missed; therefore, the pooling strategy fits better for surveillance studies [8].

It has been observed that in few cases the positive speciments presented lower Ct value when tested in pools than when tested individually. It has been hypothesised that this is due to the carrier effect of the higher RNA content in pools [7].

Overall, the results of the current study indicate that testing in pools of 5 or 6 is a reliable option for SARS-CoV-2 detection when mass testing is needed. However, laboratories are strongly recomended to validate the procedure prior application, in order to ensure the adequate performance of both the extraction and amplification assays without losing diagnostic accuracy. The procedure is especially beneficial when the prevalence of the disease is low and when it is used for surveillance studies.

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Declarations

Conflict of interest The authors declare that there is no conflict of interest.

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