




# Pooling of Nasopharyngeal Swab Samples To Overcome a Global Shortage of Real-Time Reverse Transcription-PCR COVID-19 Test Kits

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**ABSTRACT** The global outbreak and rapid spread of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) have created an urgent need for large-scale testing of populations. There is a demand for high-throughput testing protocols that can be used for efficient and rapid testing of clinical specimens. We evaluated a pooled PCR protocol for testing nasopharyngeal (NP) swabs using known positive/negative and untested clinical samples that were assigned to pools of 5 or 10. In total, 630 samples were used in this study. Individual positive samples with cycle threshold ( $C_T$ ) values as high as 33 could be consistently detected when pooled with 4 negative samples (pool of 5), and individual positive samples with  $C_T$  values up to 31 could be consistently detected when pooled with 9 negative samples (pool of 10). Pooling of up to 5 samples can be employed in laboratories for the diagnosis of COVID-19 for efficient utilization of resources, rapid screening of a greater number of people, and faster reporting of test results.

**KEYWORDS** COVID-19, COVID-19 diagnosis, PCR, pooled PCR, SARS-CoV-2

COVID-19 is caused by SARS-CoV-2, which belongs to the *Coronaviridae* family. Since the first case of human infection was reported in the Wuhan province of China in November 2019 (1), the disease has rapidly spread around the world and has been declared as a global pandemic by the World Health Organization (<https://www.who.int/>). We now know that widespread testing is needed in order to effectively respond to the COVID-19 pandemic. In order to limit the spread of the virus and to introduce appropriate quarantine measures, several countries have resorted to large-scale diagnostic testing of their populations. Mass testing for COVID-19 will be key to preventing a second wave of infections as nations and states reopen and residents move around more freely. Testing is especially critical for identifying asymptomatic individuals. However, acute shortages of testing supplies have plagued the health care system. Under these circumstances, exploring novel methods, such as sample pooling, will aid in increasing testing capacity. Nasopharyngeal (NP) and oropharyngeal swabs are the predominant specimen types being tested (<https://www.cdc.gov/coronavirus/2019-ncov/>). Other, easier-to-collect specimen types, such as saliva, are also being evaluated by different researchers (2–4). Real-time reverse transcription-PCR (rRT-PCR) has been widely employed for the diagnosis of COVID-19. In addition to individual laboratory-developed tests (3), the Centers for Disease Control (CDC), state laboratories in other countries (5), and different commercial entities (<https://www.fda.gov/medical-devices/coronavirus-covid-19-and>

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[-medical-devices/faqs-testing-sars-cov-2](#)) have successfully developed various rRT-PCR protocols targeting different genomic regions of SARS-CoV-2.

The unexpected and sudden spread of COVID-19 in the United States has created a high demand for testing needs that cannot be met by state public health laboratories alone due to a lack of availability of suitable infrastructure required for high-volume testing. In addition, heavy testing volumes across the country has put a strain on timely availability of reagents and other consumables that are essential for sample processing and testing. Pooling of samples can be helpful in addressing the current challenges of capacity and reagent availability. Sample pooling is a common approach used in the surveillance of infectious diseases such as coronavirus, influenza, tritrichomoniasis, Johne's disease, etc. in herds/flocks of animals (6–9). A similar concept of pooling has been recognized for COVID-19 diagnosis by different groups (10–17) for successful screening of samples with variable detection sensitivity.

The current pandemic is believed to have a zoonotic origin (18, 19). Coronavirus infections are common in different animal species and are known to cause severe disease outbreaks (20–22). Animal disease diagnostic laboratories across the country have developed necessary infrastructure and capacity to perform high-volume testing of different clinical specimen types in the event of such disease outbreaks. These laboratories can serve as a tremendous resource for testing of human samples during global health crises, highlighting the significance of the One Health approach in disease diagnosis (23–26). The Oklahoma Animal Disease Diagnostic Laboratory (OADDL) at Oklahoma State University (OSU) was registered under CLIA (Clinical Laboratory Improvement Amendments) regulations with the appropriate state agency for human sample testing. Samples received at OADDL were tested using a commercially available rRT-PCR kit.

In this study, we evaluated the effectiveness of pooling nasopharyngeal (NP) specimens for COVID-19 diagnosis. Samples were assigned to pools of 5 or 10 before testing, and results were compared with those of individually tested samples. Pooling of samples not only helps in increasing test volume capacity and decreasing costs, but also in improving test turnaround times and conserving valuable resources.

## MATERIALS AND METHODS

This study was approved by the Oklahoma State University Institutional Review Board (IRB) (application no. IRB-20-247).

**Sample pooling.** In total, 630 nasopharyngeal swabs in saline or viral transport media, submitted to OADDL for COVID-19 testing, were used in this study. Samples from individual swab specimens (400  $\mu$ l each sample) were pooled to give a pooled sample volume of 2 ml or 4 ml for pools of 5 and 10, respectively.

**Initial pooling evaluation with PBS or known negative samples.** Ninety swabs, determined as positive (9 swabs) or negative (81 swabs) based on previous testing at OADDL, were used for initial evaluation studies. The 9 known positive samples were diluted 10-fold in phosphate-buffered saline (PBS; pH 7.4; Hardy Diagnostics, CA) or with 9 or 4 known negative samples to make pools of 10 ( $n=9$ ) and 5 ( $n=9$ ), respectively, before RNA extraction and PCR testing.

**Pooling evaluation on previously untested (unknown) samples.** In total, 300 untested swab samples were used. Untested swab samples ( $n=170$ ) were randomly assigned to pools of 10 ( $n=17$ ) and the remaining 130 were randomly assigned to pools of 5 ( $n=26$ ) before RNA extraction and PCR testing.

**Pooling evaluation on low-viral-load samples.** In total, 240 samples were used. Twenty-four known positive samples with cycle threshold ( $C_t$ ) values of  $>28$  were tested in pools of 10 and 5. Pools contained a known positive sample mixed with 9 or 4 known negative samples to make pools of 10 ( $n=18$ ) and 5 ( $n=18$ ), respectively, before RNA extraction and PCR testing.

**RNA extraction.** RNA was extracted from 400  $\mu$ l of each individual and pooled sample using the KingFisher Flex platform (Thermo Fisher Scientific, MA) and a commercially available kit (MagMax viral/pathogen nucleic acid isolation kit; Thermo Fisher Scientific) following the FDA Emergency Use Authorization protocol provided by the manufacturer.

**Real-time PCR assay.** A commercially available multiplex rRT-PCR kit (TaqPath COVID-19 multiplex diagnostic solution; Thermo Fisher Scientific) was used for testing samples, following the FDA Emergency Use Authorization protocol provided by the manufacturer. All nasopharyngeal swab samples were tested individually, as well as in their respective pools. Eluted RNA (50  $\mu$ l) was used as the template for each 25- $\mu$ l PCR mixture; each reaction consisted of 40 amplification cycles. This kit targets 3 regions (N gene, open reading frame 1ab [ORF1ab], and S gene) of the SARS-CoV-2 genome. PCR testing was performed on ABI 7500 Fast DX and 7500 Fast platforms (Applied Biosystems; Thermo Fisher Scientific). A sample was considered "positive" when amplification of at least 2 target regions were detected. If only

**TABLE 1** Results from nonparametric statistical analysis of comparing known positive individual samples and the corresponding pools

Comparison	Target	Hodges-Lehman estimate of median $C_T$ value difference	Distribution-free 95% confidence interval <sup>a</sup>
Individual ( $n = 8$ ) vs 10-fold dilution in PBS ( $n = 8$ )	N gene	-3.47	(-1.07, -6.34)*
	ORF1ab	-3.08	(-0.65, -6.23)*
	S gene	-3.18	(-0.33, -6.06)*
Individual ( $n = 8$ ) vs pool of 10 patient samples ( $n = 8$ )	N gene	-3.33	(-0.97, -5.77)*
	ORF1ab	-2.94	(-0.99, -5.63)*
	S gene	-3.18	(-1.05, -5.62)*
Individual ( $n = 8$ ) vs pool of 5 patient samples ( $n = 8$ )	N gene	-3.09	(-0.99, -5.6)*
	ORF1ab	-2.76	(-0.5, -5.03)*
	S gene	-2.88	(-0.53, -5.21)*

<sup>a</sup>,  $P < 0.05$  (Wilcoxon's rank sum test).

one gene was detected, the result was interpreted as "inconclusive." The threshold for determination of  $C_T$  values was set around 100,000 relative fluorescence units (RFU) on the linear portion of the log amplification curve.

**Statistical analysis.** A nonparametric Wilcoxon's rank sum test was used to test for the median difference between the  $C_T$  values of the positive samples in the pool when tested individually (without pooling) and the corresponding  $C_T$  values of the different pooled samples, namely, one known positive sample diluted 10-fold in phosphate-buffered saline, one known positive sample with 9 known negative samples, and one known positive sample with 4 known negative samples. An estimate of the median difference is presented using the Hodges-Lehmann approach, along with a distribution-free 95% confidence interval (CI) calculated by the method of Moses according to Decker (27). All analyses were performed using SAS 9.4 software (SAS Institute, Inc., Cary, NC), and a  $P$  value of  $<0.05$  was considered statistically significant.

## RESULTS

Initial studies were performed using 9 known positive samples diluted in phosphate-buffered saline (PBS) or pooled with known negative samples. There was an error in the dilution and pooling of one of the known positive samples, and results from this sample was excluded from data analysis. The remaining PBS-diluted positive samples and all 16 sample pools (each pool comprising one known positive and four or nine known negative samples), tested positive by rRT-PCR. As expected, an increase in  $C_T$  values was observed following the dilution of known positive samples in the different pools (Table 1). The difference in medians of the cycle threshold ( $C_T$ ) values was statistically significant between known positive samples and samples tested in pools (Wilcoxon rank sum test;  $P < 0.05$ ). The Hodges-Lehmann estimate of the median difference and the corresponding distribution-free 95% confidence interval (CI) were calculated using the method of Moses and are presented in Table 1. Further testing was performed using unknown samples, and these results are presented in Tables 2 and 3.

Previously untested samples ( $n = 170$ ), randomly assigned to 17 pools of 10 each were tested by rRT-PCR. All 170 samples were also tested individually.  $C_T$  values of the individual positive samples making up a pool and the  $C_T$  values of their corresponding pools are shown in Table 2. Out of 170 samples, 10 samples tested positive individually. These samples formed part of 6 pools, out of which 4 pools tested positive (pools 14<sub>10</sub>, 15<sub>10</sub>, 16<sub>10</sub>, and 17<sub>10</sub>) and two pools (3<sub>10</sub> and 7<sub>10</sub>) failed to test positive. The remaining 11 pools, which did not contain any individual positive samples, tested negative. All positive pools contained at least one positive sample. Pool 14<sub>10</sub> had multiple ( $n = 5$ ) positive samples. An individual sample with  $C_T$  values as high as 33.43, 30.66, and 32.4 for the N gene, ORF1ab, and the S gene, respectively, was successfully detected when pooled (pool 15<sub>10</sub>; see Table 2) with 9 other samples. The N-gene  $C_T$  values of the positive samples that made up the two pools (3<sub>10</sub> and 7<sub>10</sub>) that failed to be detected accurately were  $>35$  when tested individually. The specificity and sensitivity of rRT-PCR testing of these unknown samples in pools of 10 were 100% and 66.67%, respectively.

Previously untested samples ( $n = 130$ ), randomly assigned to 26 pools of 5 each,

**TABLE 2**  $C_T$  values of the positive pools of 10 and corresponding individual samples making up the pool<sup>c</sup>

Pool no. <sup>d</sup>	$C_{T10p}$ (pooled PCR) for <sup>a</sup> :				Sample no. <sup>e</sup>	$C_{T1}$ (individual PCR) for <sup>b</sup> :			
	N gene	ORF1ab	S gene	$C_{T10p}$ interpretation		N gene	ORF1ab	S gene	$C_{T1}$ interpretation
3 <sub>10</sub>	<b>U</b>	<b>U</b>	U	<b>Negative</b>	2	37.12	35.13	U	Positive
					1, 3–10	U	U	U	Negative
7 <sub>10</sub>	<b>U</b>	39.05	<b>U</b>	<b>Inconclusive</b>	6	35.31	33.02	36.27	Positive
					1–5, 7–10	U	U	U	Negative
14 <sub>10</sub>	18.95	16.87	17.69	Positive	3	18.82	16.52	17.43	Positive
					5	30.56	29.14	30.42	Positive
					6	25.07	22.09	23.04	Positive
					7	16.89	15.26	15.95	Positive
					8	30.19	28.29	29.68	Positive
					1–2, 4, 9–10	U	U	U	Negative
15 <sub>10</sub>	32.19	29.62	31.34	Positive	9	33.43	30.66	32.4	Positive
					1–8, 10	U	U	U	Negative
16 <sub>10</sub>	24.6	22.43	23.78	Positive	9	31.8	30.69	32	Positive
					1–8, 10	U	U	U	Negative
17 <sub>10</sub>	28.99	26.13	27.13	Positive	2	26.97	24.24	25.23	Positive
					1, 3–10	U	U	U	Negative

<sup>a</sup> $C_{T10p}$ ,  $C_T$  values of 10-sample pools; U, undetermined  $C_T$  values.

<sup>b</sup> $C_{T1}$ ,  $C_T$  values of SARS-CoV-2-positive samples when tested individually; U, undetermined  $C_T$  values.

<sup>c</sup>Boldface indicates discordant pooled versus individual positive sample results.

<sup>d</sup>Total number of pools tested,  $n = 17$ .

<sup>e</sup>Total number of individual samples tested,  $n = 170$ .

were tested by rRT-PCR. All 130 samples were also tested individually.  $C_T$  values of the individual positive samples making up a pool and the  $C_T$  values of their corresponding pools are shown in Table 3. Out of 130 samples, 3 samples tested positive individually ( $C_T$  values < 29). Each of these samples formed part of 3 separate pools, all of which tested positive (pools 7<sub>5</sub>, 16<sub>5</sub>, and 17<sub>5</sub>). The remaining 23 pools tested negative and did not contain any samples that individually tested positive. The specificity and sensitivity of rRT-PCR testing in pools of 5 for unknown samples was 100%.

Previously tested samples ( $n = 240$ ) comprising 24 positive samples with low viral load (high  $C_T$  values;  $C_T > 28$ ) and 216 negative samples were tested in 24 pools of 5 and 24 pools of 10 (Table 4). Each pool contained one positive sample and 4 or 9 negative samples. PCR results of individual positive samples show that the N gene and ORF1ab were consistently detected for all 24 samples, and the S gene was only detected in 19 out of 24 samples. Among the 24 5-sample pools, 18 tested positive, 3 tested inconclusive, and 3 tested negative. Among the 24 10-sample pools, 11 tested positive, 2 tested inconclusive, and 11 tested negative. According to the PCR kit manufacturer recommendation, a sample is categorized as “positive” if two out of the three PCR targets are detected. Out of the 24 pools tested, the N gene, ORF1ab, and the S gene were detected in 21, 18, and 8 of the 5-sample pools, respectively, and 13, 11, and 5 of the 10-sample pools, respectively. For pools of 10 (Table 4), all three targets (N

**TABLE 3**  $C_T$  values of the positive pools of 5 and of corresponding individual samples making up the pool

Pool no. <sup>c</sup>	$C_{T5p}$ (pooled PCR) for <sup>a</sup> :				Sample no. <sup>d</sup>	$C_{T1}$ (individual PCR) for <sup>b</sup> :			
	N gene	ORF1ab	S gene	$C_{T5p}$ interpretation		N gene	ORF1ab	S gene	$C_{T1}$ interpretation
7 <sub>5</sub>	18.14	16.77	17.81	Positive	2	19.79	17.83	18.38	Positive
					1, 3–5	U	U	U	Negative
16 <sub>5</sub>	23.99	21.64	22.72	Positive	2	21.94	20.43	20.88	Positive
					1, 3–5	U	U	U	Negative
17 <sub>5</sub>	29.77	26.98	28.03	Positive	2	28.45	26.13	26.39	Positive
					1, 3–5	U	U	U	Negative

<sup>a</sup> $C_{T5p}$ ,  $C_T$  values of 5-sample pools.  $C_T$  values for 3 PCR targets (N gene, ORF1ab, and S gene) are shown.

<sup>b</sup> $C_{T1}$ ,  $C_T$  values of SARS-CoV-2-positive samples when tested individually; U, undetermined  $C_T$  values.  $C_T$  values for 3 PCR targets (N gene, ORF1ab, and S gene) are shown.

<sup>c</sup>Total number of pools tested,  $n = 26$ .

<sup>d</sup>Total number of individual samples tested,  $n = 130$ .

**TABLE 4** Pooling study using individual positive samples with  $C_T$  values of  $>28^a$

Pool no.	$C_{Ti}$ (individual positive sample PCR) for <sup>b</sup> :			$C_{T5p}$ (pooled PCR) for <sup>c</sup> :			$C_{T5p}$ interpretation	$C_{T10p}$ (pooled PCR) for <sup>d</sup> :			$C_{T10p}$ interpretation
	N gene	ORF1ab	S gene	N gene	ORF1ab	S gene		N gene	ORF1ab	S gene	
1	29.59	29.22	29.87	32.07	31.26	31.81	Positive	33.02	32.72	33.08	Positive
2	29.9	28.93	29.65	32.34	31.37	32.1	Positive	33.44	32.35	33.21	Positive
3	30.39	28.73	29.47	32.5	31.31	31.84	Positive	34.15	32.06	33	Positive
4	30.4	30.03	31.75	28.43	29.07	30.46	Positive	33.35	33.05	<b>U</b>	Positive
5	30.68	29.76	30.34	32.81	31.93	32.87	Positive	34.3	32.62	33.34	Positive
6	30.8	29.28	29.95	34.24	33.15	34.03	Positive	36.49	33.53	35.81	Positive
7	31.04	29.76	30.46	32.89	33.75	33.87	Positive	36.71	34.15	<b>U</b>	Positive
8	31.23	32.57	33.16	32.16	32.93	36.5	Positive	<b>U</b>	<b>U</b>	<b>U</b>	<b>Negative</b>
9	31.3	30.1	31.43	36.62	35.74	<b>U</b>	Positive	38.2	35.55	<b>U</b>	Positive
10	31.37	32.31	33.43	35.73	38.13	<b>U</b>	Positive	<b>U</b>	<b>U</b>	<b>U</b>	<b>Negative</b>
11	32.21	32.11	37.69	34.35	38.9	<b>U</b>	Positive	35.26	37.17	<b>U</b>	Positive
12	32.26	32.05	36.36	34.57	36.57	<b>U</b>	Positive	36.43	<b>U</b>	<b>U</b>	<b>Inconclusive</b>
13	32.35	32.67	36.42	35.25	35.03	<b>U</b>	Positive	<b>U</b>	<b>U</b>	<b>U</b>	<b>Negative</b>
14	32.59	31.22	33.09	35.12	35.11	<b>U</b>	Positive	<b>U</b>	<b>U</b>	<b>U</b>	<b>Negative</b>
15	33.74	36.07	<b>U</b>	35.08	37.93	<b>U</b>	Positive	39.79	37.18	<b>U</b>	Positive
16	33.86	33.99	36.65	33.99	37.2	<b>U</b>	Positive	38.11	<b>U</b>	<b>U</b>	<b>Inconclusive</b>
17	33.95	34.49	36.01	36.42	<b>U</b>	<b>U</b>	<b>Inconclusive</b>	<b>U</b>	<b>U</b>	<b>U</b>	Negative
18	34.5	33.31	37.41	37.21	34.72	<b>U</b>	Positive	35.95	37.63	<b>U</b>	Positive
19	34.66	33.79	37.62	37.91	<b>U</b>	<b>U</b>	<b>Inconclusive</b>	<b>U</b>	<b>U</b>	<b>U</b>	<b>Negative</b>
20	35	35.49	<b>U</b>	<b>U</b>	<b>U</b>	<b>U</b>	<b>Negative</b>	<b>U</b>	<b>U</b>	<b>U</b>	<b>Negative</b>
21	35.46	33.74	35.57	<b>U</b>	<b>U</b>	<b>U</b>	Negative	<b>U</b>	<b>U</b>	<b>U</b>	<b>Negative</b>
22	36.58	35.33	<b>U</b>	38.38	<b>U</b>	<b>U</b>	<b>Inconclusive</b>	<b>U</b>	<b>U</b>	<b>U</b>	<b>Negative</b>
23	36.59	36	<b>U</b>	38.06	34.16	<b>U</b>	Positive	<b>U</b>	<b>U</b>	<b>U</b>	<b>Negative</b>
24	38.62	35.01	<b>U</b>	<b>U</b>	<b>U</b>	<b>U</b>	<b>Negative</b>	<b>U</b>	<b>U</b>	<b>U</b>	<b>Negative</b>

<sup>a</sup>U,  $C_T$  value undetermined. A sample was considered "positive" when amplification of at least 2 target regions was detected. If only one gene was detected, the result was interpreted as "inconclusive." Boldface indicates discordant pooled versus individual positive sample results.

<sup>b</sup> $C_{Ti}$ ,  $C_T$  values of SARS-CoV-2-positive samples when tested individually.

<sup>c</sup> $C_{T5p}$ ,  $C_T$  values of 5-sample pools containing the individual positive sample.

<sup>d</sup> $C_{T10p}$ ,  $C_T$  values of 10-sample pools containing the individual positive sample;

gene, ORF1ab, and S gene) were consistently detected when the N-gene  $C_T$  values of the individual positive samples making up the pool were  $<30$ . The S gene could not be detected consistently in pools of 10 when the N-gene  $C_T$  values of the individual positive samples were  $>30$ . When the N-gene  $C_T$  values of the individual positive samples were  $>31$ , ORF1ab and the N gene failed to be consistently detected after pooling. In pools of 5 (Table 4), all three genes were consistently detected when N-gene  $C_T$  values of the individual positive samples added to the pool were  $<31$ . The S-gene, ORF1ab, and N-gene targets could not be detected consistently in pools of 5 when the N-gene  $C_T$  values of the individual positive samples making up the pool were  $>31$ ,  $>33$ , and  $>35$ , respectively.

**DISCUSSION**

Pooling of nasopharyngeal (NP) samples for SARS-CoV-2 testing has been found to be a promising strategy for high-volume testing (10, 12, 14, 28). Mathematical models have also been proposed to estimate optimal pooling size (29). Studies employing different pool sizes have been successfully attempted (10, 12, 28). Our study shows that pooling NP samples in groups of 5 is more reliable than pooling in groups of 10. Detection of samples with low  $C_T$  values ( $C_T < 28$ ; higher viral load) was consistently achieved when diluted 10-fold in pristine buffer or when pooled in groups of 5 or 10 with other clinical samples. However, there was a higher chance of false negatives when samples with high  $C_T$  values (lower viral loads) were pooled and tested. High  $C_T$  values were more often detected when tested in pools of 5 rather than pools of 10.

Generally, pooling should result in an increase in  $C_T$  value due to the dilution of positive samples. However, we observed in some instances that the  $C_T$  values of positive pools were lower than those of individual positive samples (e.g., Table 4, pool no. 4;

Table 2, pool 14<sub>10</sub>). This could be attributed to sampling and/or testing (platform/PCR mix) variations and possibly dilution of PCR inhibitory factors. Additionally, samples used for this study were collected from different places in Oklahoma without a consistent volume or type of viral transport medium. These, along with other factors, such as swab type and temperature, can also affect viral nucleic acid detection by PCR (30). Lohse et al. (12) hypothesized that the carrier effect of increased RNA content of the pools may be a reason for lower  $C_T$  values in pooled samples.

Targeting multiple genes in a single test improves the specificity of diagnostic testing (31). The commercial kit used in this study targeted 3 different regions of the viral genome (N gene, ORF1ab, and S gene). No false positives were detected in our study when samples were pooled in groups of 5 or 10, indicating a test specificity of 100%. Compared to the S-gene and ORF1ab PCR targets, the N gene was most reliably detected in the individual and pooled positive samples tested. Postpooling increases in  $C_T$  values among the 24 high- $C_T$ -value known positive samples (Table 4), for the N gene, ORF1ab, and the S gene ranged from  $-1.97$  to  $5.32$  (average,  $2.19$ ),  $-0.96$  to  $6.79$  (average,  $2.79$ ), and  $-1.29$  to  $4.08$  (average,  $2.3$ ), respectively, when samples were pooled in groups of 5, and  $2.95$  to  $6.9$  (average  $4.19$ ),  $2.86$  to  $5.45$  (average  $3.70$ ), and  $3$  to  $5.86$  (average  $3.8$ ), respectively, when pooled in groups of 10. This wide range of  $C_T$  values may be a result of variations in the composition of the pooled matrix that contains samples from different patients. Positive pools (detection of two gene targets) were consistently identified when the N-gene  $C_T$  value of the individual positive sample making up a pool was  $<33$  for pools of 5 and  $<31$  for pools of 10. Suspect/inconclusive pools (at least one gene target detected) could be consistently identified when the N-gene  $C_T$  value of the individual sample making up the pool was  $<35$  for pools of 5 and  $<31$  for pools of 10. These results are consistent with those of another study (16) and suggest that smaller pool sizes will help in improving sensitivity of detection.

Several pooling strategies and PCR protocols have been studied for COVID-19 diagnosis (12, 13, 16, 17). In most studies, samples were pooled before RNA extraction; however, pooling of extracted RNA has also been reported for COVID-19 testing (14, 32). Compared to direct sample pooling, RNA pooling does not save as much in terms of extraction reagents. Nevertheless, RNA pooling will be helpful in attaining a faster test turnaround time by avoiding the need to perform additional extractions if positive pools are detected and samples in the pool have to be tested individually. Direct pooling of clinical samples has been more commonly studied to evaluate different pooling sizes and PCR protocols. The pooling of 6 or fewer samples in a clinical setting was found to be helpful to avoid false-negative results due to high  $C_T$  values (33). Another interesting study carried out at Sanya Airport in China for screening incoming population recommended pooling of 10 samples (34). Lohse et al. (12) studied pool sizes of 4 to 30 and were successful in identifying a sample with a  $C_T$  value as high as 34 in a pool of 30 samples by targeting S and E genes. Ben-Ami et al. (17) and Lim et al. (13) reported detection of samples with  $C_T$  values of  $>37$  in pools of 8 and 10, respectively. Ben Ami et al. (17) followed a protocol targeting S and E genes, and Lim et al. (13) targeted the *RdRp* gene, which has been reported to be more sensitive for pooling-based diagnostics (15). Overall, our results are consistent with those of other studies and show that pooling is a viable approach for COVID-19 diagnosis. Lack of consistency in detecting high- $C_T$ -value samples remains a major limitation of pooling-based diagnostics.

Another important factor to be considered before adopting pooling strategies is the disease prevalence rate. We evaluated hypothetically the effect of pooling samples, using known data from the first 100 samples that tested positive during four different months of 2020. To get to the first 100 positive samples, we tested 2,620, 2,197, 3,767, and 1,280 total samples for the months of April, May, June, and July 2020. Thus, the percent positivity rates during this period were 3.8, 4.54, 2.65, and 7.81, respectively. If all of the samples were to be randomly pooled in groups of 5, for each of the months of April, May, June, and July, we would have had 90, 65, 91, and 88 pools, respectively, containing at least one positive sample. Out of these, 18, 9, 22, and 16 pools for the

months of April, May, June, and July, respectively, would have contained one positive sample with a  $C_T$  value greater than our cutoff value of 33 and most likely would have been reported as a false negative. This would have altered our percent positivity rates to 3.12, 4.14, 2.07, and 6.56 for the respective months from April to July. These results indicate that at the tested prevalence rates, pooling can cause a false decrease in positivity rate by 8.8% to 21.9%. A study from Nebraska, USA (10), reported that testing NP samples in pools of 5 was an efficient strategy for COVID-19 diagnostics at a 5% disease prevalence rate. This agrees with our observations on pool size. If pooling strategies are employed for diagnostic applications, all samples that make up a pool should be individually tested to identify positive patients once a pool is identified as positive. In our study, positive samples that comprised pool 14<sub>10</sub> (Table 2) were received from counties with high disease incidence rate per data from the Oklahoma State Department of Health. Pooling of samples from such regions is not ideal for diagnostic purposes, since it will require individually testing samples of several pools to identify positive patients. This in turn slows down the diagnostic workflow and potentially increases resource utilization. When large number of samples are pooled, use of sub-pooling strategies may help in reducing the number of individual samples to be tested once a positive pool is identified (12). However, based on results of our studies, pooling of large number of samples is not recommended. Pooling techniques are best employed in areas of low COVID-19 prevalence in order to increase the efficiency, cost basis, and test turnaround time.

**Conclusions.** Pooling of samples can be considered a viable option in high-volume laboratories for COVID-19 diagnosis. Data from our study demonstrate that pooling of up to 5 samples is more reliable for diagnostic purposes. Testing of pooled samples can also be used for surveillance purposes in low-prevalence areas or samples from members of a household. Moreover, such pooling strategies will also help in addressing the worldwide paucity of testing resources.

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