Asymptomatic Patient Testing After 10:1 Pooling Using the Xpert Xpress SARS-CoV-2 Assay

Gary W. Procop, MD, MS,[1] Marion Tuohy, MT(ASCP),[1] Christine Ramsey, MT(ASCP),[1] Daniel D. Rhoads, MD,[1] Brian P. Rubin, MD, PhD,[1] and Richard Figler, MD[2]

From the [1]Pathology and Laboratory Medicine Institute and [2]Sports Medicine, Cleveland Clinic, Cleveland, OH.

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ABSTRACT

Objectives: *Pool testing for severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) preserves testing resources at the risk of missing specimens through specimen dilution.*

Methods: *To determine whether SARS-CoV-2 specimens would be missed after 10:1 pooling, we identified 10 specimens with midrange (ie, 25-34 cycles) and 10 with late (ie, >34-45 cycles) crossing threshold (Ct) values and tested these both neat and after 10:1 pooling. Final test results and Ct changes were compared.*

Results: *Overall, 17 of 20 specimens that contained SARS-CoV-2 were detected after 10:1 pooling with the Xpert Xpress SARS-CoV-2 Assay (Cepheid), rendering an 85% positive percentage of agreement. All 10 of 10 specimens with an undiluted Ct in the mid-Ct range were detected after 10:1 pooling, in contrast to 7 of 10 with an undiluted Ct in the late-Ct range. The overall Ct difference between the neat testing and the 10:1 pool was 2.9 cycles for the N2 gene target and 3 cycles for the E gene target. The N2 gene reaction was more sensitive than the E gene reaction, detecting 16 of 20 positive specimens after 10:1 pooling compared with 9 of 20 specimens.*

Conclusions: *An 85% positive percentage of agreement was achieved, with only specimens with low viral loads being missed following 10:1 pooling. The average impact on both reverse transcription polymerase chain reactions within this assay was about 3 cycles.*

Key Points

- Specimens with midrange crossing threshold values will still be detected after 10:1 pooling, when a highly sensitive reverse transcription polymerase chain reaction (RT-PCR) assay is used for testing.
- The effect of 10:1 dilutional pooling on the N2 gene and E gene RT-PCRs in the Xpert Xpress severe acute respiratory syndrome coronavirus 2 assay was 2.9 and 3.0 cycles, respectively.
- Only some specimens from patients with a low viral load (ie, late crossing threshold values) were not detected following 10:1 pooling; the impact of missed specimens will vary based on clinical factors but should be thoroughly considered.

The coronavirus disease 2019 (COVID-19) pandemic has overwhelmed the testing capabilities in the United States.[1] The health care community was initially taxed with developing and validating tests and implementing rapid-cycle, reverse transcription polymerase chain reaction (RT-PCR) assays to detect severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) to accurately diagnose symptomatic patients with COVID-19. Soon after, these tests were used for screening members of high-risk, vulnerable populations (eg, members of congregate living facilities), preoperative patients, patients admitted to the hospital, and first responders. Most recently, the Centers for Disease Control and Prevention called for the testing of asymptomatic individuals who have had close contact with a person with documented SARS-CoV-2 infection.[2] In addition, there have been increasing demands to perform surveillance screening for asymptomatic individuals for activities including participating in team sports activities to going back to school. These additional testing demands have further stressed a system that is already significantly challenged with reagent, equipment,

and personnel shortages and timely test result delivery.[3] In response to these overwhelming demands, pooling of selected specimens has been considered as a potential solution to preserve resources without significantly reducing viral-detection capabilities.

We undertook a study to evaluate the feasibility of detecting specimens known to contain SARS-CoV-2 using the Xpert Xpress SARS-CoV-2 Assay (Cepheid) following 10:1 pooling. The Xpert Xpress SARS-CoV-2 assay is a highly sensitive and specific SARS-CoV-2 assay that is easy to use and provides results in about an hour. The automated nature of this assay has afforded the performance of SARS-CoV-2 testing in a wide variety of settings, including those without onsite expertise in molecular diagnostics. These features have placed this assay in high demand at our institution and elsewhere, challenging the manufacturer's production capabilities. The successful pooling of specimens to be tested with the Xpert Xpress SARS-CoV-2 assay would expand the availability of this useful assay. There was not an emergency use authorization (EUA) for the pooling of specimens using the Xpert Xpress SARS-CoV-2 assay at the time this study was performed. This study was performed, in part, to determine whether 10:1 specimen pooling assessed by the Xpert Xpress SARS-CoV-2 assay would meet the acceptability criteria for pooling set forth by the US Food and Drug Administration (FDA). The manufacturer or any user intending to perform pooling using this or any SARS-CoV-2 assay would need to undertake the appropriate FDA EUA submission and review before use on clinical specimens.

We studied pooling in a group of asymptomatic patients with COVID-19, following institutional review board approval. We chose this population because there had been a recent increase in requests to test asymptomatic individuals (eg, presurgical testing), and the positivity rate in this group in our population was known to be low (ie. approximately 0.5%), which is optimal for pooling.[3]

Materials and Methods

The nasopharyngeal swab specimens included in this study were from patients who were designated as asymptomatic by their health care provider at the time the test order was placed and were collected in 0.9% saline transport media. There is a significant restriction on the ordering of SARS-CoV-2 tests for individuals without symptoms consistent with COVID-19 at our institution. These individuals are usually presurgical candidates or admissions to the medical intensive care unit, labor and delivery, or behavioral health units. The patient specimens were initially determined to contain SARS-CoV-2 using one of 4 nucleic acid amplification assays that had been given EUA status by the FDA. These specimens were then retested using the Xpert Xpress SARS-CoV-2 assay, so as to assign the neat or undiluted specimen into either a moderate crossing threshold (Ct; ie, 25-34 cycles) or a late Ct threshold category (ie, >34-45 cycles), with the intention of studying the effect of pooling for 10 specimens in each of these categories. We supposed that specimens with very high viral loads (ie, very early Ct values) would likely be detected following 10:1 pooling. Therefore, we excluded these specimens from the study and concentrated on specimens with moderate viral loads (ie, Ct values from 25-34 cycles) and those with low viral loads (ie, Ct >34-45 cycles).

The negative specimens that were used for each of the 10:1 pools consisted of a 300-µL aliquot from each of 9 specimens that individually tested negative with the Xpert Xpress SARS-CoV-2 assay. These pools were made in duplicate so that one of the pools could be tested for SARS-CoV-2 using the Xpert Xpress SARS-CoV-2 assay to ensure the absence of the target virus. Otherwise stated, the specimens that were used for pooling were tested both individually and after pooling and shown not to contain the SARS-CoV-2 virus.

Each of the 10:1 pools consisted of one 300-µL aliquot from a SARS-CoV-2–positive specimen and nine 300-µL aliquots from 9 unique SARS-CoV-2–negative patient specimens. These specimens were tested immediately using the Xpert Xpress SARS-CoV-2 assay. Testing of both the neat specimen and the 10:1 pool was performed on the same day. The qualitative test results and an assessment of the Ct differences between the neat and 10:1 pool results were compared for each of the RT-PCR assays included in this test [Table 1].

Results

Overall, 17 of 20 positive specimens that were pooled 10:1 were detected with the Xpert Xpress SARS-CoV-2 assay, rendering an 85% positive percentage agreement between the undiluted (ie, neat) specimen and the 10:1 pool, which meets the positive percentage agreement required by the FDA. All specimens that had an undiluted Ct within the mid-Ct range (ie, 25-34 cycles) were detected after 10:1 pooling.

When specimens were evaluated without dilution (ie, neat), the N2 gene target was detected in 19 of 20 specimens (95% target detection) with a mean Ct of 37.1 cycles, whereas the E gene was detected in 17 of 20 specimens

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Table 1
Ct Changes of the Xpert Xpress SARS-CoV-2 Target Genes Following 10:1 Pooling

	E Gene			N2 Gene		
Specimen	Original Ct	10:1 Pool Ct	Ct Difference	Original Ct	10:1 Pool Ct	Ct Difference
Midrange[a]						
1	29.8	32.7	2.9	32.8	36.3	3.5
2	31.4	36.1	4.7	ND	ND	NA
3	33.1	ND	NA	35.4	40.4	5.0
4	32.8	39.6	6.8	35.5	40.5	5.0
5	30.5	ND	NA	33.9	43.2	9.3
6	30.7	37.3	6.6	34.7	38.5	3.8
7	30.0	33.3	3.3	32.8	36.9	4.1
8	32.4	ND	NA	35.4	39.2	3.8
9	30.1	33.5	3.4	33.2	37.1	3.9
10	33.0	ND	NA	36.2	39.6	3.4
Late range[b]						
11	41.5	ND	NA	39.5	41.8	2.3
12	35.2	ND	NA	39.0	ND	NA
13	ND	ND	NA	41.7	43.0	1.3
14	ND	ND	NA	43.0	ND	NA
15	35.8	38.3	2.5	36.4	41.3	4.9
16	ND	ND	NA	39.1	41.1	2.0
17	40.3	ND	NA	41.0	ND	NA
18	35.0	41.8	6.8	38.0	39.5	1.5
19	42.3	41.8	−0.5	38.6	40.5	1.9
20	38.3	ND	NA	38.9	41.7	2.8

Ct, crossing threshold; NA; not available; ND, not detected; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2.
[a]Midrange: 25-34 cycles.
[b]Late range: >34-45 cycles.

(85% target detection), with a mean Ct of 34.2 cycles. After 10:1 pooling of these specimens, the N2 gene target was detected in 16 of 20 (80% target detection) with a mean Ct of 40.0 cycles, whereas the E gene was detected in 9 of 20 specimens (45% target detection) with a mean Ct of 37.2 cycles. The overall Ct differences between the neat and 10:1 pool tests were 2.9 cycles for the N2 gene target and 3 cycles for the E gene target and were mathematically consistent with the expected changes from a 10:1 dilution. The RT-PCR reaction for the detection of the N2 gene was more sensitive than the reaction for the E gene, detecting 16 of 20 positive specimens after 10:1 pooling compared with 9 of 20 specimens.

Analysis of the 2 RT-PCR reactions, stratified by the neat Ct value (ie, the mid- or late Ct range), was undertaken. Within the mid-Ct range, the N2 gene target was detected in 9 of 10 neat specimens (mean Ct = 34.4 cycles) and 9 of 10 specimens pooled 10:1 (mean Ct = 39.1 cycles). The mean Ct difference for the N2 gene target due to 10:1 pooling of specimens in the mid-Ct ranges was 4.7 cycles. Within the mid-Ct range, the E gene target was detected in 10 of 10 neat specimens (mean Ct = 31.4 cycles) and 6 of 10 specimens pooled 10:1 (mean Ct = 35.4 cycles). The mean Ct difference for the E gene target in the mid-Ct range due to 10:1 pooling was 4.0 cycles.

Within the late-Ct range, the N2 gene target was detected in 10 of 10 neat specimens (mean Ct = 39.5 cycles) and 7 of 10 specimens pooled 10:1 (mean Ct = 41.3 cycles). The mean Ct difference for the N2 gene target in the late Ct category due to 10:1 pooling was 1.8 cycles. Within the late-Ct range, the E gene target was detected in 7 of 10 neat specimens (mean Ct = 38.3 cycles) and 3 of 10 specimens pooled 10:1 (mean Ct = 40.6 cycles). The mean Ct difference for the E gene target in the late-Ct range due to 10:1 pooling was 2.3 cycles.

Discussion

The pooling of samples has been used for many years in the public health setting. This approach is particularly useful for determining the prevalence of an infectious agent in a large population. Rohde described the use of this technique in the public health setting to test vast quantities of mosquitoes for the detection of medically important arboviruses.[4] The use of pooling moved closer to the patient care setting with the use of pool testing to screen donated blood products for HIV and hepatitis B virus.[4-6] However, pool testing is not a practice commonly, if ever, encountered in the patient care–centered medical laboratory in the United States, until now.

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The COVID-19 pandemic has overwhelmed the public health and medical communities, necessitating the rationing of resources, including those needed for medical tests. Medical testing reagents, as well as equipment and materials (eg, plastics for plates and pipette tips) have been in limited supply.[1,3] The ability of laboratories to test patient specimens is directly related to the availability and allocation of testing materials from commercial vendors. These limitations have necessitated the explorations of alternative specimen types, such as saliva to address shortages in swabs and personal protective equipment, and the pooling of specimens to make optimal use of the limited supply of diagnostic testing materials.[3,7,8] Although the rationing of testing and the exploration of alternative specimens and methods may be appropriate, these should be evaluated in a scientifically sound manner with a clear delineation of the advantages and disadvantages of these approaches.

The primary advantage of pool testing is to extend the use of the limited supply of reagents, as described previously.[9] When the prevalence of infection is low, then a pool of many patient specimens may be assessed with a single test rather than multiple independent tests, thereby conserving test reagents and supplies.[3] However, new operational workflows and information system solutions are needed to successfully implement diagnostic testing using pooled specimens, as this workflow has not been developed previously or used for other analytes. When a pool tests positive, then the pool must be deconstructed and each of the specimens that constituted the pool tested independently to determine which specimens were positive and which were negative. As the prevalence of disease increases, however, the frequency in which a pool tests positive and must be deconstructed increases and, depending on the prevalence, can actually increase the use of materials and labor and the likelihood of human error (eg, contamination and specimen misidentification).[3] Consequently, the optimal use of pooling depends on both the prevalence of disease and the context in which pooling is used.

The Clinical Laboratory Standards Institute defines the limit of detection as the target concentration for which an assay can reproducibly detect that target 95% of the time. It stands to reason that if a target is present in a patient specimen at or near the limit of detection and that specimen is diluted through pooling, then such specimens may not be detected. In other words, pooling specimens dilutes the individual specimens, which decreases the ability of the assay to detect the positive specimen with a low concentration of target.

Abdalhamid et al[10] studied 5:1 pooling (ie, 1 positive specimen combined with 4 negative specimens) and demonstrated a change in Ct between 0 and 5 cycles, which is similar to the findings of our study, although we pooled 10:1. They suggest pooling as a viable option for diagnostic testing when the incidence rate of SARS-CoV-2 is 10% or less. They also estimate that in such a setting, the overall increase in testing capacity could be more than 69%.[10] Yelin and colleagues[11] investigated various pooling strategies and suggest that a single positive sample may be detected in pools of up to 32 specimens with a false-negative rate of 10%. In our study, a 10:1 pool resulted in an overall false-negative rate of 15%; importantly, however, the only specimens that were not detected were those with low viral loads (ie, high Ct values).

These findings raise important questions. What is an acceptable false-negative rate, and what is the risk of not detecting an individual with a low viral load (ie, a high Ct value)? These questions should be considered within the individual clinical context and in light of the emerging information concerning the prolonged test positivity in COVID-19 convalescence and the decreased likelihood of transmission in convalescence.[12,13]

The viral load in patients with COVID-19 increases after infection and peaks just before the onset of symptoms.[14] Consequently, there is a risk, albeit small, of missing an asymptomatic or presymptomatic patient very early in disease, which is an important consideration in situations such as presurgical screening and for hospital cohorting. The mischaracterization of a positive patient as negative could be devastating if a patient were to be admitted to a COVID-19–negative unit or preparing for organ transplantation. Conversely, in the outpatient setting, the risk of missing a patient with a low viral load may be minimal if the patient were notified of the possibility of the false-negative result and informed to maintain recommended mitigation strategies and to contact their provider if they were to become symptomatic. The recognized extended period of test positivity in convalescence with minimal to no risk of transmission could be problematic for individuals who have recovered from the disease but need to be tested for travel, return to work or school, or another function. Failing to detect such individuals by pooling would have little to no consequence if these individuals adhere to the recommended mitigation strategies (eg, appropriate masking).

Conclusions

We report the effect of 10:1 pooling on specimens with initial (ie, neat) Ct values in the mid-Ct (25-34 cycles) and late Ct (>34-45 cycles) ranges. All specimens with an initial Ct value in the mid-Ct range, which represent patients with enhanced potential for spread, were detected following 10:1 pooling. These findings support the feasibility of pooling specimens in a low-prevalence setting as a means of conserving testing resources in the appropriate

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clinical context with the preservation of appropriate mitigation strategies.

Corresponding author: Gary W. Procop, MD, MS; procopg@ccf.org.

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References

1. Kaul KL. Laboratories and pandemic preparedness: a framework for collaboration and oversight. *J Mol Diagn.* 2020;22:841-843.
2. Centers for Disease Control and Prevention. Overview of testing for SARS-CoV-2 (COVID-19). 2020. https://www.cdc.gov/coronavirus/2019-ncov/hcp/testing-overview.html. Accessed October 4, 2020.
3. Binnicker MJ. Challenges and controversies related to testing for COVID-19. *J Clin Microbiol.* 2020.
4. Rohde R. COVID-19 pool testing: is it time to jump in?. https://asm.org/Articles/2020/July/COVID-19-Pool-Testing-Is-It-Time-to-Jump-In. Accessed October 4, 2020.
5. Dinesha TR, Boobalan J, Sivamalar S, et al. Occult HBV infection in HIV-infected adults and evaluation of pooled NAT for HBV. *J Viral Hepat.* 2018;25:718-723.
6. Boobalan J, Dinesha TR, Gomathi S, et al. Pooled nucleic acid testing strategy for monitoring HIV-1 treatment in resource limited settings. *J Clin Virol.* 2019;117:56-60.
7. Chen JH, Yip CC, Poon RW, et al. Evaluating the use of posterior oropharyngeal saliva in a point-of-care assay for the detection of SARS-CoV-2. *Emerg Microbes Infect.* 2020;9:1356-1359.
8. Cheuk S, Wong Y, Tse H, et al. Posterior oropharyngeal saliva for the detection of SARS-CoV-2. *Clin Infect Dis* 2020. doi:10.1093/cid/ciaa797.
9. Garg J, Singh V, Pandey P, et al. Evaluation of sample pooling for diagnosis of COVID-19 by real time-PCR: a resource-saving combat strategy. *J Med Virol.* 2020. doi:10.1002/jmv.26475.
10. Abdalhamid B, Bilder CR, McCutchen EL, et al. Assessment of specimen pooling to conserve SARS CoV-2 testing resources. *Am J Clin Pathol.* 2020;153:715-718.
11. Yelin I, Aharony N, Tamar ES, et al. Evaluation of COVID-19 RT-qPCR test in multi sample pools. *Clin Infect Dis.* 2020;71:2073-2078.
12. La Scola B, Le Bideau M, Andreani J, et al. Viral RNA load as determined by cell culture as a management tool for discharge of SARS-CoV-2 patients from infectious disease wards. *Eur J Clin Microbiol Infect Dis.* 2020;39:1059-1061.
13. Wolfel R, Corman VM, Guggemos W, et al. Virological assessment of hospitalized patients with COVID-2019. *Nature.* 2020;581:465-469.
14. Benefield AE, Skrip LA, Clement A, et al. SARS-CoV-2 viral load peaks prior to symptom onset: a systematic review and individual-pooled analysis of coronavirus viral load from 66 studies. *medRxiv.* 2020. doi:10.1101/2020.09.28.20202028.

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