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Pooling nasopharyngeal swab specimens to increase testing capacity for SARS-CoV-2

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Abstract:

The recent emergence of SARS-CoV-2 has lead to a global pandemic of unprecedented proportions. Current diagnosis of COVID-19 relies on the detection of SARS-CoV-2 RNA by RT-PCR in upper and lower respiratory specimens. While sensitive and specific, these RT-PCR assays require considerable supplies and reagents, which are often limited during global pandemics and surge testing. Here, we show that a nasopharyngeal swab pooling strategy can detect a single positive sample in pools of up to 10 samples without sacrificing RT-PCR sensitivity and specificity. We also report that this pooling strategy can be applied to rapid, moderate complexity assays, such as the BioFire COVID-19 test. Implementing a pooling strategy can significantly increase laboratory testing capacity while simultaneously reducing turnaround times for rapid identification and isolation of positive COVID-19 cases in high risk populations.

Introduction:

In December 2019, an outbreak of pneumonia with unknown origin began in Wuhan city, the capital of Hubei province in China[1]. The following month, Chinese researchers had isolated a novel coronavirus, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), from

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26 patients with viral pneumonia[2]. Pneumonia associated with SARS-CoV-2 was later designated
27 as coronavirus disease 2019 (COVID-19) by the World Health Organization in February 2020[3].
28 It was determined that after a zoonotic transmission event in Wuhan city[4], widespread person-
29 to-person transmission quickly occurred that led to the infection and death of over 80,000 and
30 3,000 people in China, respectively. To date, according to the WHO, there have been 4,258,666
31 reported cases of COVID-19, including 294,190 deaths worldwide[5].
32 Since the initial outbreak in China, COVID-19 has been declared a global pandemic affecting at
33 least 216 other countries, territories or areas. To monitor and diagnose COVID-19, the US Food
34 and Drug Administration (FDA) approved an emergency use authorization (EUA) for the CDC
35 2019-nCoV Real-Time RT-PCR Diagnostic Panel on February 4, 2020[6]. This protocol allows for
36 the rapid detection of SARS-CoV-2 RNA from clinical specimens such as, nasopharyngeal and
37 oropharyngeal swabs, sputum, bronchoalveolar lavage, and tracheal aspirates. As evidenced by
38 the ongoing SARS-CoV-2 pandemic, increased demand for testing can overwhelm diagnostic
39 laboratories and lead to drastic shortages in supplies and reagents. A strategy to overcome
40 high testing demand is to pool specimens before RNA extraction, test pools, and then retest
41 individual specimens from positive pools. Similar strategies have shown to increase testing
42 capacity for the detection of common infectious diseases such as influenza, HIV, Hepatitis, and
43 *Chlamydia trachomatis*[7–11].
44 In this study, we examined the feasibility of pooling nasopharyngeal swab specimens submitted
45 for COVID-19 testing using the CDC 2019-nCoV RT-PCR diagnostic panel without compromising
46 clinical sensitivity. Our data shows that pooling respiratory samples during times of increased

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47 volume and low disease prevalence can save time and reagents without significant
48 modifications to laboratory infrastructure or workflow.
49

50 **Methods:**

51 This study was determined to meet the exempt criteria listed in 32CFR219.104(d) from the
52 Landstuhl Regional Medical Center Exempt Determination Official.
53 During an outbreak cluster of SARS-CoV-2 in Stuttgart, Germany, 494 nasopharyngeal (NP)
54 swabs were collected and placed into 1.0 ml of normal saline. Specimens were submitted to
55 the Virology laboratory at Landstuhl Regional Medical Center for routine SARS-CoV-2 testing
56 using the CDC 2019-nCoV RT-PCR assay. Post clinical testing, specimens were de-identified and
57 randomly assigned into pools of 10 to create 50 distinct pools (the 50th pool contained 4
58 specimens diluted in 0.6 ml of transport media). Pools were created by combining 100 ul of
59 each specimen to create 1.0 ml pools. Viral transport media was added to each pool at a 1:1
60 ratio for nucleic acid extraction performed on the Roche MagNA Pure 24 platform using the
61 MagNA Pure 24 Total NA Isolation kit (Roche). Elution volume was set to 50 ul to concentrate
62 viral RNA. Each round of extraction contained a human specimen control to monitor for PCR
63 inhibition and specimen quality.
64 Detection of SARS-CoV-2 was performed using the CDC RT-PCR COVID-19 assay, which contains
65 primers and Taqman probes for two specific regions of the SARS-CoV-2 nucleocapsid (N) gene
66 and the human Rnase-P (RP) gene, which is used as an internal positive control for human
67 nucleic acid. PCR was performed according to the CDC protocol using the TaqPath 1-Step RT-
68 qPCR Master Mix, CG kit (Life Technologies) on the Applied Biosystems (ABI) 7500 Fast real-

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69 time PCR system. PCR results were interpreted as recommended in the CDC RT-PCR COVID-19
70 instructions for use. A pool was considered positive if the C_T was less than 40. Detection of
71 SARS-CoV-2 in pooled samples using the BioFire COVID-19 Test was performed according to the
72 manufacturer's instructions for use on the BioFire FilmArray 2.0 and FilmArray Torch systems.
73 All statistical analyses were conducted using Graphpad Prism 6.0.
74

75 **Results:**

76 The prevalence for individual clinical samples was 4% (19/494) for SARS-CoV-2 RNA. Among the
77 pooled samples, 30% (15/50) were positive for SARS-CoV-2 RNA, while the remaining 70%
78 (35/50) did not have detectable levels of SARS-CoV-2 RNA (**Table 1**). We observed one
79 inconclusive RT-PCR result in our pooled analysis as defined by amplification of only a single
80 SARS-CoV-2 target. In this case, the N2 target for pool 41 failed to amplify while N1 was
81 detected with a relatively high C_T (37.3). There were no invalid reactions in our analysis, as
82 defined by reactions where Rnase-P failed to amplify. Out of the 15 positive pools, 4 pools
83 contained 2 positive specimens, while the remaining 11 pools contained only 1 positive
84 specimen (**Table 2**). The mean C_T value and standard deviation for N1 and N2 of the pools were
85 29.2 (4.4) and 29.4 (4.3), respectively. Similarly, the mean C_T values of individual positive
86 specimens were 28.0 (4.5) and 29.9 (4.8) for N1 and N2, respectively. Despite dilution, there
87 was no significant difference in mean C_T value between the pooled and individually tested
88 specimens (**Figure 1**).
89 To determine if a pooling approach is feasible with rapid, moderate complexity tests, we tested
90 the 15 SARS-CoV-2 positive pools and 15 of the SARS-CoV-2 negative pools using the recently

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91 released BioFire COVID-19 Test. The BioFire COVID-19 test is a nested multiplexed RT-PCR test
92 that automates all aspects of nucleic acid testing including sample preparation, extraction, and
93 PCR, and which can detect SARS-CoV-2 within a single nasopharyngeal swab specimen in under
94 60 minutes. As expected, there was perfect agreement between the CDC 2019-nCoV RT-PCR
95 and BioFire COVID-19 assays (kappa=1.0). SARS-CoV-2 RNA was detected in all 15 positive
96 pools, whereas SARS-CoV-2 RNA was not detected in all 15 of the negative pools using the
97 BioFire COVID-19 Test (**Table 3**).
98

99 **Discussion:**

100 We found that a single NP swab specimen containing SARS-CoV-2 RNA can be consistently
101 detected in a pool of 10 samples. Our data shows an estimated false negative rate of
102 approximately 7% (1 out of 15), although this pool was inconclusive (the N2 primer failed to
103 amplify) and was treated as a positive pool. Unlike other pooling strategies that pool purified
104 RNA extracts[12,13], our method utilized pooling clinical specimens prior to RNA extraction, which
105 removes the extraction bottleneck and allows running an endogenous internal control to
106 monitor extraction quality.

107 A linear increase in threshold cycle is expected as specimens are pooled, however, we did not
108 observe a significant change in C_T values for either primer pair in our pooled samples. Given
109 that PCR efficiency of each primer pair can differ, any inconclusive result for a pool should be
110 treated as positive and individually tested. Case in point, the only inconclusive result in our
111 study was found in pool 41, where the N2 target failed to amplify. This pool contained 2
112 positive specimens and one inconclusive specimen. This suggests there may have been PCR

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113 inhibitors present in the individual sample that carried over to the pooled specimen resulting in
114 an inconclusive result. Both positive specimens in pool 41 had relatively high C_T values. In our
115 lab, specimens with high C_T values are commonly observed in convalescent patients 14-30 days
116 after symptomatic infection, and do risk escaping detection when combined in larger pools due
117 to loss of sensitivity. It should also be noted that a negative pool result would not differentiate
118 between a true negative and an inconclusive or invalid result due to improper sample collection
119 or storage. Given the clinical performance of this and other published pooling protocols[7,12,13], it
120 is possible that larger pools could be used with further RT-PCR optimization to allow lower
121 detection limits for low-concentration RNA.
122 Disease prevalence should also be taken into consideration when implementing a pooling
123 strategy. Recently, Noriega and Samore used a Bayesian modeling approach to show testing
124 throughput more than doubles when prevalence rates are ≤8%, and this occurs with optimal
125 pool sizes between 4 and 12 samples. Conversely, as prevalence increases, they show
126 improvements in testing throughput diminishes significantly[14]. During this surveillance period,
127 SARS-CoV-2 prevalence was determined to be approximately 4% (19/494), which is ideal for
128 pool sizes of 10. In this study, we found that 30% (15/50) pools were positive for SARS-CoV-2,
129 which equates to 200 individual extractions and RT-PCR reactions (50 pools and 150
130 individuals), representing a 60% savings in extractions and RT-PCR reactions, which is significant
131 during times of surge testing and in limited-resource situations.
132 Recently, numerous rapid molecular diagnostic platforms have received an Emergency Use
133 Authorization from the FDA. These include low to moderate complexity assays from BioFire,
134 Cepheid, and Abbott that can detect SARS-CoV-2 in approximately 1 hour[15,16]. Using the

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135 recently released BioFire COVID-19 Test, we found that this platform could reliably detect a
136 single positive sample in pools of up to 10 specimens, with equal rates of detection as the CDC
137 COVID-19 RT-PCR assay. This is not surprising given the published limits of detection for the
138 CDC COVID-19 RT-PCR and BioFire COVID-19 test are in the range of 10^2 RNA copies/ml. These
139 results support the use of rapid molecular diagnostic platforms for routine disease surveillance
140 of critical working groups such as healthcare providers and military units, where large-scale
141 quarantines can have grave consequences.
142 In summary, we show that a pooled-sample strategy can augment a laboratory's testing
143 capability and relieve extreme pressure from limited resource situations without sacrificing RT-
144 PCR sensitivity and specificity. Importantly, a pooling strategy can reduce turnaround times for
145 prompt identification and isolation of infected individuals to effectively curb the transmission of
146 COVID-19 and other infectious disease outbreaks.
147

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153

154 **Declarations of Interests:**
155 None
156

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Table 1. RT-PCR C_T values of pooled specimens positive for SARS-CoV-2

Positive Pool	Ct Values		
	N1	N2	Rnase-P
2	28.6	29.7	31.8
8	25.4	26.2	25.7
11	35.9	38.7	33.7
12	29.7	31	32.5
13	30.7	32.1	31.4
15*	29.5	30.3	30.1
18	27.1	27.6	25.2
19	20.7	20.9	25.1
20*	26.3	26.8	25.2
22	29.5	30.3	30.1
25	30	29.3	25.8
41*†	37.3	Und.	26.5
43*	23.5	23.5	27
47	30.9	31.3	26.2
50	33.6	33.3	26.2

* Pool containing 2 positive specimens

† Inconclusive RT-PCR result

Und.: Undetected

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Table 2. RT-PCR C_T values of individual specimens positive for SARS-CoV-2

Pool	Sample	Ct Values		
		N1	N2	Rnase-P
2	2696	25.2	26.2	27.6
8	2610	24.7	26.1	28.6
11	2535	31	34.7	25.9
12	2697	23.9	34.9	30.4
13	2624	21.1	21.9	28.3
15	2595	29.3	30.6	29.5
15	2620	26.3	31.6	29.1
18	2586	29.4	32.1	26.7
19	2803	20.4	21.3	27.9
20	2665	30	31.5	28.6
20	2785	27.5	29	25.6
25	2662	29.5	30.6	27.8
41	3010	33.5	32.2	28
41	2975	36.1	36	27.1
43	3186	35	38.4	29.3
43	3202	24.2	25.3	32.1
47	3164	30.2	31.8	30
50	3104	31.3	30.5	22.9
22	2497	23.2	23.4	26.4

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Table 3. CDC 2019-nCoV RT-PCR and BioFire COVID-19 comparator analysis

		BioFire COVID-19 Test	
		Positive	Negative
CDC 2019-nCoV RT-PCR	Positive	15	0
	Negative	0	15

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Figure 1. Comparison of mean C_T value between positive pooled and individually tested samples. Data are represented as the mean ± standard error the mean.