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LETTER TO THE EDITOR

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Pooling of nasopharyngeal swab specimens for SARS-CoV-2 detection by RT-PCR

To the Editor,

Until an effective vaccine is available, interruption of community circulation of severe acute respiratory syndrome-coronavirus (SARS-CoV-2) is crucial to control virus spread. To this end, systematic testing of large population groups by reverse transcription polymerase chain reaction (RT-PCR) is mandatory to case identification and contact tracing, thereby minimizing the likelihood of resurgence in contagion.[1] This approach faces a variety of obstacles, most notably the limited availability of reagents resources. Sample pooling for RT-PCR has been effectively used for screening of blood donors for human immunodeficiency virus-1 and hepatitis C virus in low-prevalence setttings.[2] This strategy has also been applied to detect community transmission of SARS-CoV-2 in the United States early in the pandemic, when virus circulation was low.[3] Nevertheless, sample pooling may decrease the sensitivity of RT-PCR assays due to specimen dilution. The objective of this study was to assess whether pooling of 5 or 10 nasopharyngeal specimens (NP) specimen allowed detection of individual SARS-CoV-2 positive samples spanning a large range of cycle thresholds (C_t) values as determined by a sensitive commercially available RT-PCR (REALQUALITY RQ-2019-nCoV from AB ANALITICA; Padua, Italy, performed on the Applied Biosystems 7500 instrument). This RT-PCR assay targets the *E* (envelope) and *RdRp* (RNA-dependent RNA polymerase) genes of SARS-CoV-2 in a single reaction, with limit of detections of 125 and 150 copies/mL, respectively (according to the manufacturer). The current study was approved by the Ethics Committee of Hospital Clínico Universitario INCLIVA.

NP specimens collected with flocked swabs in 3 mL of universal transport medium (Becton Dickinson, Sparks, MD), which had been stored at −80°C, were retrieved for analysis. A total of 30 leftover specimens that tested negative for SARS-CoV-2 were mixed and used for pooling. In turn, 10 RT-PCR positive NP specimens yielding C_t values ranging from 23.4 to 38.8 for the *E* gene, and 21.8 to 35.8 for the *RdRP* gene were selected for the experiments. A total of 20 minipools containing either 5 (n = 10) or 10 (n = 10) samples were made, each of which included a unique positive NP specimen (Table 1). These latter specimens had been collected from adult

TABLE 1 Detection of SARS-CoV-19 RNA by RT-PCR in pooled nasopharyngeal specimens from patients with COVID-19

***E* (Envelope) gene**			***RdRp* (RNA polymerase, RNA-dependent) gene**		
Nonpooled (C_t)	Positive NP specimen pooled with four negative NP specimens (C_t)	Positive NP specimen pooled with nine negative NP specimens (C_t)	Nonpooled (C_t)	Positive specimen pooled with four negative NP specimens (C_t)	Positive NP specimen pooled with nine negative NP specimens (C_t)
23.4	25.5	26.2	21.8	24.1	27.1
23.9	26.0	27.3	23.6	27.6	26.2
27.2	27.8	28.2	26.5	27.2	28.3
27.3	27.5	28.6	26.3	28.6	29.7
31.6	35.4	35.3	30.7	36.8	35.2
31.9	35.5	40.0	34.5	38.0	40.0
35.9	ND	ND	35.1	37.4	44.7
35.9	ND	ND	35.8	ND	ND
38.2	ND	ND	36.4	ND	ND
38.8	44.0	ND	35.2	43.4	ND

Note: A volume of 45 and 22.5 µL/specimen was used for preparing (manually) minipools of 5 and 10 samples, respectively, to achieve a final volume of 225 µL, which was then mixed (1:1) with lysis buffer. RNA extraction was performed using the DSP virus Pathogen mini kit on the QiaSymphony Robot instrument (Qiagen, Valencia, CA). RT-PCR was performed and interpreted according to the manufacturer instructions.

Abbreviations: C_t, cycle threshold; NP, nasopharyngeal exudate.

patients (median age, 61.5 years; range, 30-85) presenting with mild disease and not requiring hospitalization, at a median of 5 days (range, 1-7 days) following appearance of symptoms.

As shown in Table 1, positive NP specimens were detected in minipools of both sizes, as long as undiluted samples yielded RT-PCR C_{ts} < 32 for the *E* gene (6 out of 10) or <35.2 for the *RdRp* gene (7 out of 10). As expected, C_{ts} were reached later in pooled samples. In contrast, most NP samples displaying RT-PCR C_{ts} > 35.8 for the *E* gene or 35.7 for the *RdRP* gene remained undetected in minipools of five specimens (3/4 and 2/3, respectively) or in minipools of 10 samples (4/4 and 3/3, respectively). Thus, our data thus seemed to suggest that targeting two SARS-CoV-2 genes may increase the efficiency of detection of positive specimens in low-size minipools. This extent needs to be confirmed though.

Comprehensive testing policies in the community are primarily aimed at identifying SARS-CoV-2-infected asymptomatic or paucisymptomatic individuals, who are known to represent a major source of transmission.[1] Pooling strategies of RT-PCR testing may be advantageous when compared to assaying individual samples separately if the proportion of positive specimens in the set of samples is low enough (~1%).[4] Previously reported data suggested that a single positive specimen could be detected in pools of up to 32 samples,[5] with a false-negative rate of around 10%. In that work, pure RNA instead of original specimens were pooled for RT-PCR reactions.[5] Likewise, the identification of cases was achieved in pools of 48 samples including 1 to 5 RT-PCR positive samples.[6] Our experience was less satisfactory, yet, positive specimens that went undetected when tested in pools presumably had very low viral loads, as inferred by RT-PCR CT values, most likely <6 $\log_{10}$ RNA copies/swab, which was previously shown to represent the viral RNA load threshold for virus infectivity[7]; it is uncertain whether missing such type of positive results would have consequences in terms of public health.

It is possible that the efficiency of our approach may have been improved had fresh specimens been used for analyses, since degradation of RNA in samples during storage or thawing could have occurred. In summary, our data indicated that pooling NP specimens for RT-PCR testing may result in false-negative results in patients presenting with mild COVID-19. Although speculative, the procedure evaluated herein may be improved by pooling larger volumes of each individual specimen, or perhaps by collecting samples directly in a small volume of lysis buffer, provided that specimens are processed without delay.

In a scenario of gradual de-escalation from COVID-19 lockdown, early case detection and thorough contact tracing will eventually impact on success. To this end, a large number of RT-PCR test will have to be performed. Modeling studies suggest that adapting pooling number set-up based on prevalence of cases would be well-suited to systematic testing of asymptomatic and mild cases.[8] Given the important benefit in terms of reagent savings inherent to this strategy, further studies are warranted to appraise its applicability in routine community surveys.

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CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

AUTHOR CONTRIBUTIONS

IT and EA performed the experiments and collected the data. DN analyzed the data and wrote the manuscript. All authors reviewed and approved the final version of the manuscript.

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