Letter to the Editor

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Upper respiratory samples pooling for screening SARS-CoV-2 infection: ready for the prime time?

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To the Editor,

Coronavirus disease 2019 (COVID-19), the pandemic disorder caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), has already infected over 30 million people, causing nearly 1 million worldwide casualties. Despite the death rate of COVID-19 is now estimated at around 3.0%, thus lower than the two previous coronavirus pathologies severe acute respiratory syndrome (SARS; ~9.6%) and Middle-East respiratory syndrome (MERS; ~34.4%), the much broader diffusion of SARS-CoV-2 is contributing to magnify the burden of morbidity and mortality all around the world [1].

The high infectious potential of SARS-CoV-2, as well as the considerable burden of virus transmission by asymptomatic and/or pre-symptomatic individuals, has placed laboratory diagnostics at the very core of almost each preventive and containment strategy [2]. Early molecular diagnosis, identifying the presence of SARS-CoV-2 RNA in upper respiratory specimens through nucleic acid amplification tests (NAATs), is the cornerstone for index case isolation and thoughtful contact tracing, which are effective means for breaking the chain of transmission. This strategy has been fully endorsed by the World Health Organization (WHO), as reflected by the well-known mantra "test, test, test" [3]. Unlike other infectious pathologies, however, the number of people infected with SARS-CoV-2 is enormous and is still exponentially growing, thus requiring considerable testing escalation, with a notable risk of jeopardizing laboratory responsiveness. A recent survey of the American Association of Clinical Chemistry (AACC) has emphasized that as many as 40% worldwide laboratories were unable to obtain necessary supplies to cover all COVID-19 tests in early August 2020, with nearly two thirds of these experiencing shortage of staff, reagents and test kits, and one third highlighting difficulties in purchasing swabs [4]. This concerning worldwide picture, which does not save rich and highly industrialized countries such as the US (shortage of testing capacity was as high as 50% in this country), requires development and implementation of alternative strategies designed for optimizing testing capabilities without disrupting diagnostic accuracy. Sample pooling is one potential approach, conventionally intended as the process of pooling clinical specimens (e.g., upper respiratory samples) and then analyzing the pools with NAATs, reserving later individual samples testing to those pools being cumulatively positive for SARS-CoV-2 [5]. Some published reports have confirmed that, under some specific environmental and analytical conditions, this may be a feasible strategy for counteracting local shortage of reagents or limited test availability, especially in extensive testing areas, where throughput could be enhanced with contextual reduction of turnaround time and costs.

In a seminal study, Cherif and colleagues theoretically estimated that the optimal strategy for pooling nasopharyngeal samples encompasses the generation of pools with 5–13 individual clinical specimens in a real life scenario, where prevalence of COVID-19 is between 1 and 10% and reverse transcription polymerase chain reaction (RT-PCR) is characterized by at least 1100 RNA copies/mL sensitivity [6]. In a subsequent study, Abdalhamid et al. generated 25 experimental pools mixing some negative nasopharyngeal specimens with one SARS-CoV-2 positive nasopharyngeal sample, and showed that the optimal pool size (i.e., that combining improved test efficiency with unvaried diagnostic accuracy) encompassed the analysis (SARS-CoV-2 *N1* and *N2* genes) of pools made with five individual clinical specimens [7]. Overall, this improved testing efficiency was associated with ~70% saving of staff time and reagents. Similar evidence has been published by Kim et al., who generated pools of variable size by mixing 50 individual upper respiratory specimens collected from SARS-CoV-2

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infected patients and 300 similar specimens collected from SARS-CoV-2-negative subjects [8]. Overall, the authors found that pools made of up to six specimens retained 100% diagnostic sensitivity (SARS-CoV-2 *E* or *RdRP* genes), whilst those generated with more samples increased testing efficiency but displayed lower diagnostic sensitivity. A clinical validation of small-size pools for screening SARS-CoV-2 infection has been carried out by Petrucca et al. [9]. Briefly, the authors generated several 5-sample pools, which were tested for the SARS-CoV-2 *N* and *ORF1ab* genes along with their corresponding individual naso-oral pharyngeal samples, showing 100% diagnostic sensitivity and specificity. In the local area, with relatively low COVID-19 prevalence (i.e., <2%), the diagnostic accuracy of the 5-pool strategy was found to be excellent, and samples pooling was also characterized by shorter turnaround time (<24 h) and considerable cost saving (e.g., up to 70% of the total expenditure needed for individual sample analysis). The important impact in terms of resource saving has also been emphasized by Garg et al. [10], who showed that the analysis of SARS-CoV-2 *E* and *RdRP* genes in 5-specimen pools in areas with low to moderate prevalence (i.e., ~5%) displayed unvaried accuracy, but enabled to save up to 80% of reagents needed for individual specimens testing. Interestingly, the 5-nasopharyngeal specimens pool strategy has also been validated by Farfan and colleagues [11], who showed that the cycle threshold (CT) value (SARS-CoV-2 *ORF1ab* gene) in the pools was adequate using techniques based on automated or manual viral RNA extraction, whilst the analytical sensitivity was found to be remarkably decreased using no extraction assays, thus raising doubts as to whether some rapid molecular tests could be used for testing pools. An intriguing approach has then been proposed by Regen and colleagues [12], who developed a specific formula (i.e., Pool size = $[1.24] \times [\text{Prevalence}]^{-0.466}$) for estimating the theoretical size of pools based on local disease prevalence. Using this equation, that obviously needs to be clinically validated, it could be calculated that the optimal pool size in areas with disease prevalence of 1, 2, 5 and 10% would encompass mixing 11, 8, 5 and 4 individual samples. Other group testing algorithms have been proposed, as recently reviewed by Bilder and colleagues [13].

Evidence that sample pooling may be regarded as a valuable perspective for offsetting SARS-CoV-2 testing escalation has also been provided using other biological matrices. Pasomsub et al. recently demonstrated that saliva pools, encompassing from 5 to 10 separate samples [14], allowed detecting both SARS-CoV-2 *ORF1ab* and *N* genes with optimal accuracy. In particular, the median CT value in the 5-sample pools did not significantly differ from that of the individual specimens.

Table 1: Ad interim, evidence-based indications for upper respiratory tract samples pooling for screening severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection.

– Only used in areas with low disease prevalence (e.g., <5%)
– For population screening and not for diagnosing SARS-CoV-2 infection in highly suspected or symptomatic cases
– Prepared by mixing upper respiratory specimens (or saliva) and not extracted RNA
– Molecular assay shall be validated for this purpose
– Methods encompassing RNA extraction are preferred
– Five is the optimal individual sample size for preparing pools
– Presence of interfering substances must be ruled out
– Accurate (automatic) traceability to individual samples must be assured
– A second aliquot of clinical specimens shall be available for individual testing when pool screening is positive

Taken together, the available data on upper respiratory samples pooling for screening SARS-CoV-2 infection would allow to draw some ad interim conclusions, summarized in Table 1. First and foremost, this strategy shall only be used in areas with limited prevalence of SARS-CoV-2 infection (i.e. typically <5%), for population screening and not for diagnostic investigation of highly suspected or symptomatic cases. The pools shall then be prepared using clinical specimens, preferably nasopharyngeal samples (or saliva), and must not be made with extracted viral RNA. It is then extremely important that the local NAAT has been thoughtfully validated for purposes of pool testing, thus displaying adequate analytical sensitivity, and preferably encompassing RNA extraction rather than employing rapid molecular assays. As concerns the number of individual specimens, current evidence would support the use of pools made of not more than five upper respiratory tract samples, since this value seems to combine significantly improved efficiency with unvaried diagnostic sensitivity. The presence of interfering substances (e.g., antiviral drugs, cell-free hemoglobin, and so forth) shall always be ruled out, traceability (preferably automatic) to individual samples must be ensured and, finally, a second aliquot shall always be available for being individually tested when pool screening is positive.

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