Original Article

Sensitivity and efficiency of RNA sample pooling for real-time quantitative polymerase chain reaction testing for SARS-CoV-2

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Background: In spite of the worth of pool testing in public health, data on the sensitivity and efficiency of real-time quantitative polymerase chain reaction (RT-qPCR) pool testing for the diagnosis of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection in middle and low-income countries are limited.

Methods: We mixed single specimens of extracted RNA positive for the SARS-CoV-2 envelope (E) gene by RT-qPCR with negative specimens, in pools of 4 (n=89), 8 (n=92), 16 (n=102), and 32 (n=105) specimens each. We estimated the average change in cycle threshold (Ct) for each pool size and added it to the Ct values of the first 1,350 tests in our lab, to obtain dilution-corrected Ct values. We estimated pool sensitivity as the proportion of samples with dilution-corrected Ct >40, and used it in simulations of the efficiency (tests used/true case detected) of binary split pool testing.

Results: We tested 388 pools. Average Ct changes were 2.21, 2.51, 3.27, and 3.94 cycles, for pools of 4, 8, 16, and 32 specimens, respectively. Corresponding pool tests sensitivities were 91.1%, 89.6%, 85.8% and 82.5%. Pool testing was substantially more efficient than individual testing. For prevalence of 0.5% to 2.0%, the efficiency of pools of ≥8 specimens was 30% to 280% higher, and the number of people tested was 4.4 to 13.9 times higher than those of individual testing.

Conclusions: Binary split pool testing substantially increases the number of people tested and the number of true cases detected per test used. This strategy is key to curtail the transmission of SARS-CoV-2, by increasing efficiency in the identification and isolation of symptomatic and asymptomatic infected individuals.

Keywords: Polymerase chain reaction (PCR); pool testing; COVID-19; mass screening; severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2); Colombia; testing efficiency

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Introduction

The health burden resulting from the outbreaks of COVID-19 in middle-low and low-income countries (ML-LIC) has been staggering. As of Nov 12 2020, ML-LIC had experienced 23.4% of all cases (about 11.5 million) and 22.0% of all deaths in the world [1,185,331] (1). Even though they has helped curtail the magnitude of the COVID-19 pandemic, most of the world population still have very limited access to effective vaccines, and COVID-19 is becoming an endemic disease in rich and poor countries (2-5). The need for booster shots, the emergence of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) variants of concern, and the lack of accessible effective therapeutics contribute to the need of access to accurate testing in ML-LIC (6-8). Widespread access to COVID-19 testing to detect individuals with asymptomatic infections is key to contain the spread of the virus, decrease mortality, and avoid substantial economic losses in ML-LIC countries and poor populations (9).

Mass testing for SARS-CoV-2 infection makes feasible the identification and isolation of symptomatic and asymptomatic infectious individuals (10), and has been key in stopping the spread of the virus in many countries (11). Faced with limited testing capacity, ML-LIC countries have made limited use of real-time quantitative polymerase chain reaction (RT-qPCR) tests for preventing SARS-CoV-2 transmission (12). Indeed, official country level data (13) indicate the ratio of RT-qPCR tests per person has been 11 times higher in high income countries (6.7) than in ML-LIC countries (0.6), and the number of tests per confirmed case has been 38% higher (17.5 *vs.* 12.7, respectively). In order to save testing resources, several countries use a protocol based on the detection of the envelope (E) gene as a single test target for the diagnosis of SARS-CoV-2 infection (14-19).

To test as many people as possible, with limited resources, it is possible to conduct pooled tests for groups of people, rather than individuals, and conduct further testing in the individuals included in positive pools. Pool testing could be used as a screening approach for disease surveillance, expeditious studies of outbreaks, and in groups of people sharing the same environment. For instance, widespread testing of health care workers could be achieved by weekly testing using pool testing, regardless of their history of exposure to SARS-CoV-2, a task may not be feasible in resource limited setting (20,21).

Pool testing has been scarcely used for SARS-CoV-2 infection diagnosis in ML-LIC countries. This is in large part due to a lack of data on the sensitivity and efficiency of RT-qPCR pool testing, as compared to individual testing, particularly when the E gene is used as the single test target for diagnosis. We evaluated the sensitivity of RT-qPCR tests in RNA pools of 4, 8, 16, and 32 specimens, using the SARS-CoV-2 E gene as a single target, and assessed the relative efficiency of pool testing to detect true positive (TP) cases of infection. We show that pool testing is several times more efficient than individual testing to diagnose, identify, and isolate infected individuals, particularly if they are asymptomatic. We present the following article in accordance with the STARD reporting checklist (available at https://jphe.amegroups.com/article/view/10.21037/jphe-21-97/rc).

Methods

We used respiratory samples from the Central Research Laboratory (CRL), Universidad Industrial de Santander (UIS), Bucaramanga, Colombia, collected on July-September 2020, from symptomatic and asymptomatic individuals, being tested for clinical care or epidemiologic surveillance. The study was approved by the UIS' and the University of Wisconsin-Madison's Institutional Review Boards (IRB00003739). The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). No informed consent was required, since the study was based on respiratory samples stored before the start of our study.

We selected specimens of extracted RNA from SARS-CoV-2 RT-qPCR positive tests, independently of their cycle threshold (Ct). Single positive specimens were mixed with 3, 7, 15, and 31 negative specimens to make pools of 4, 8, 16, and 32 samples each. Pools of extracted RNA samples were used, instead of pre-extraction pools of nasopharyngeal swaps, because they had been stored and were readily available. Before including an originally positive sample in a pool, the sample was re-tested to confirm it was still positive and to get a new Ct value for that sample. The sample was included in a pool only if it was positive in this second individual test.

We tested at least 89 pools of each size, and ensured that about 25% of pools of each size had a positive specimen with a low viral load (Ct >34 cycles), to match the proportion of specimens with low viral load in the first 1,350 tests conducted in our lab. A Ct >34 cycles was pre-defined, because a dilution of a RT-qPCR positive sample

mixed with 31 additional negative samples would result, on average, in an increase of five Ct cycles. This would increase the false negative rate for pools of 32 specimens, since the resulting Ct value would approach the cut-point of 40 Ct cycles.

RT-qPCR testing

Nasopharyngeal swabs and tracheal aspirates were collected and transported following a standard protocol (22). RNA was extracted using MagMAX™ Viral/Pathogen II (MVP II) Nucleic Acid Isolation Kit (Thermo Fisher Scientific, Waltham, MA, USA), according to the manufacturer's instructions. Purified RNA was stored at −80 ℃ in Micro Amp 96-well real-time optical PCR plates, until testing, 1 to 2 weeks later. A pool mix was prepared using 5 µL of purified RNA for final volumes of 20, 40, 80, and 160 µL for pools of size 4, 8, 16, and 32 samples, respectively. Five µL from the pool mix were used for RT-qPCR analysis, following the Berlin Protocol (23). A 25-µL reaction was set up containing 5 µL of RNA, 12.5 µL of 2× reaction buffer, 1 µL of reverse transcriptase/Taq mixture from Superscript III one-step RT-qPCR system (Invitrogen, Waltham, MA, USA), and 0.4 µL of a 50 mM magnesium sulfate solution. The test uses one primer and probe to detect a region in the SARS CoV-2 E gene at the concentration recommended by developers (23). Thermal cycling was performed at 55 ℃ for ten minutes for reverse transcription, followed by 95 ℃ for 3 min and then 45 cycles of 95 ℃ for 15 s, and 58 ℃ for 30 s. Positive and negative controls were included in each test. Tests with a Ct ≤40 cycles were considered positive.

Statistical analysis

We assumed viral RNA would have been detected in negative samples, if additional amplification cycles were used. Therefore, to prevent potential bias resulting from assigning a value of 40 Ct to these samples (24), we treated their Ct values as a right-censored variable, missing at random, conditionally on the Ct of the individual positive sample included in the pool. We used interval regression to obtain imputed values as low as 40 and as high as 42 Ct cycles, for negative samples, and generated 100 complete datasets (24,25).

We computed the average change in Ct value as the difference (Δ) between the individual Ct of the positive sample in the pool, the first time the individual sample was tested, when received in our lab, and the Ct of its pool, and its standard deviation (SD_Δ). We took a random value (Ct_r) of the distribution of Δ for each pool, normal (Δ, SD_Δ), and added it to the Ct value of each of the first 1,350 positive samples in our lab (Ct_{0i}) to obtain dilution-corrected Ct values. We calculated the sensitivity for each pool size ($Sens_{pool}$) as the proportion of the original 1,350 samples with a dilution-corrected Ct value (Ct_{0i} + Ct_r) >40 cycles (19). This process was repeated 1,000 times for each pool size, and the median and the upper and lower 2.5% of the simulated distribution were taken as Senspool and its 95% confidence interval (CI), respectively. This analysis was also conducted using the Ct value from the positive sample in the pool, tested at the time when the pool including that sample was also tested.

We wrote a program in Stata (StataCorp.; 2017; Stata Statistical Software: Release 15; College Station, TX: StataCorp LLC) to conduct simulations of pool testing by binary splitting and evaluate the efficiency of pool testing as compared to individual testing. We used $Sens_{pool}$ for poll testing and ($Sens_{ind}$) of 95% (26,27) and specificity ($Spec_{ind}$) of 99.5% (26) for individual testing. For nested pools, we assumed a sensitivity of 100%. Efficiency was defined as the ratio of tests used per TP cases detected for individual (E_{ind}) and pool testing (E_{pool}) (28). And relative efficiency as (E_{pool}/E_{ind})/3 (18,27,29). We took a third of the relative efficiency because we used pools of RNA, instead of pools of respiratory samples, and the amplification phase comprised a third of the total cost of a RT-qPCR test.

Results

We analyzed 388 SARS-CoV-2 positive and 5,696 negative specimens. Positive specimens had an average Ct of 26.6 cycles (95% CI: 25.8, 27.3) and 22% to 25% of all pools had one positive sample with a Ct >34 cycles (*Table 1*). The mean Ct in pooled samples was 29.6 cycles (95% CI: 28.9, 30.3), and was homogeneous across pools of different sizes. In contrast, the average change in Ct was 3.16 cycles (95% CI: 2.82, 3.50), and ranged from 2.21 in pools of 4 to 3.94 in pools of 32 samples.

The mean Ct value in the first 1,350 positive tests in our lab, used to estimate Senspool, was 27.9 cycles (95% CI: 27.5, 28.3), and 25.4% of them (95% CI: 22.9%, 28.0%) had a Ct >34 cycles. $Sens_{pool}$ estimated from dilution-corrected Ct values, ranged from 82% in pools of size 32 to 91% in pools of size 4, and were similar, but considerably more precise, than to those based on the proportion of positive pools (*Table 2*).

Table 1 Mean and 95% CI for the change in Ct value from the individual to the pooled RT-qPCR tests, by pool size

Pool size	Number of pools	Ct >34 in positive sample (%)	Mean Ct from pooled tests (95% CI)*	Ct change (SD)*	95% CI
32	105	24.8	30.8 (29.4, 32.2)	3.94 (2.40)	(3.03, 4.84)
16	102	24.5	29.8 (28.4, 31.3)	3.27 (1.92)	(2.65, 3.89)
8	92	22.8	29.3 (27.8, 30.8)	2.51 (1.50)	(2.14, 2.88)
4	89	22.5	28.3 (26.6, 29.9)	2.21 (1.37)	(1.92, 2.50)

*, Ct values from negatives tests were obtained by multiple imputation with minimum and maximum Ct of 40 and 42, respectively. CI, confidence interval; Ct, cycle threshold values; RT-qPCR, real-time quantitative polymerase chain reaction; SD, standard deviation.

Table 2 Pool sensitivity estimates based on the observed percentage of positives and on the expected change in Ct by pool size

Pool size	Sensitivity	
	Observed % positive (95% CI)	Based on Ct change* (95% CI)
32	86.7 (78.6, 92.5)	82.5 (81.5, 83.6)
16	84.1 (75.8, 90.8)	85.8 (84.8, 86.8)
8	90.2 (82.2, 95.4)	89.6 (88.7, 90.5)
4	91.0 (83.0, 96.0)	91.1 (90.1, 91.9)

*, Ct values from negatives tests were obtained by multiple imputation with minimum and maximum Ct of 40 and 42, respectively, and average change in Ct was added to the Ct values from the first 1,350 positive tests in our lab. Ct, cycle threshold values; CI, confidence interval.

The efficiency of individual testing increased with the prevalence of infection. For a doubling of the prevalence, efficacy increased by 100%: from 212, to 106, to 53 tests used per TP case detected, for prevalence of 0.5%, 1%, and 2%, respectively (*Table 3*). Pool testing efficacy also increased with prevalence, but the increase diminished with higher prevalence. For instance, for pools of 32 samples, increases in prevalence from 0.5% to 1% and 1% to 2% resulted in a 31% and 22% higher efficiency, respectively. Moreover, the smaller the pool size the higher the increase in efficiency associated to higher prevalence. Indeed, for pools of size 4, increases in prevalence from 0.5% to 1% and 1% to 5% improved efficiency by of 87% and 80%, respectively.

Although the relative gain in efficiency associated with higher prevalence was smaller for pool testing than from individual testing, pool testing was several times more efficient than individual testing in all scenarios, excepting pools of size 4. The efficiency of pools of size 8, 16, and 32 was 2.0 to 3.8, 1.7 to 2.5, and 1.3 to 1.5 times higher than that of individual testing, for prevalence of 0.5%, 1% and 2%, respectively. In contrast, the efficiency of pools of size 4 was similar to that of individual testing, regardless of the level of prevalence.

Using pool testing largely reduced the number of tests needed to detect the same number of TP cases that would have been detected using a fixed number of individual tests. For instance, if 1% of the population were infected, 20,000 tests would be needed to identify 190 TP cases, compared to about 2,600 tests if pools of size 16 were used (*Table 3*). In addition, pool testing resulted in a large increase in the number of individuals tested in a population, for a fixed number of available tests, regardless of baseline prevalence of infection. The larger the pool size, the higher the relative increase in the number of individuals tested. For instance, 20,000 tests used in pools of size four would allow testing 3.1, 3.4, and 3.8 times more people, for prevalence of 2%, 1%, and 0.5%, than individual testing. This allows detecting very large numbers of true negative, in addition to TP cases, by pool testing.

Findings from the analysis comparing the Ct from the test conducted in the positive sample when the sample was included in a pool and the Ct from the pool tests were virtually identically to those from the analysis using the original (first) Ct value for the positive sample.

Table 3 Number of true cases detected, tests used, and efficiency of individual and pool testing (tests used per TP case detected), by starting pool size and prevalence of SARS-CoV-2 in the population being tested*

Prev	Pool size	Individual testing strategy			Pool testing strategy				
		Number of tests available, (a)	True positive detected, (b)	Tests used per true positive (efficiency), (a/b)	Tests needed to identify (b) true positives, (c)	Population tested with 20,000 tests	True positives detected, (d)	Tests used per true positive (efficiency), (c/d)	Relative efficiency (95% CI)†, [(a/b)/(c/d)]/3
0.005	32	20,000	95	211.6	1,440.0	278,019	78.8	18.3 (18.2, 18.4)	3.8 (3.8, 3.9)
0.005	16	20,000	95	211.3	1,942.1	278,019	81.9	23.7 (23.6, 23.9)	3.0 (2.9, 3.0)
0.005	8	20,000	95	211.4	3,061.3	130,669	85.8	35.7 (35.5, 35.9)	2.0 (2.0, 2.0)
0.005	4	20,000	95	211.6	5,457.7	73,292	86.5	63.2 (62.7, 63.6)	1.1 (1.1, 1.1)
0.01	32	20,000	190	105.6	2,187.2	183,003	156.0	14.0 (14.0, 14.1)	2.5 (2.5, 2.5)
0.01	16	20,000	190	105.6	2,599.1	153,938	163.5	15.9 (15.8, 16.0)	2.2 (2.2, 2.2)
0.01	8	20,000	190	105.7	3,585.0	111,581	170.6	21.0 (20.9, 21.1)	1.7 (1.7, 1.7)
0.01	4	20,000	190	105.7	5,812.2	68,822	172.0	33.8 (33.6, 34.0)	1.0 (1.0, 1.0)
0.02	32	20,000	380	52.8	3,612.2	110,813	314.9	11.5 (11.4, 11.5)	1.5 (1.5, 1.5)
0.02	16	20,000	380	52.8	3,837.0	104,274	326.7	11.7 (11.7, 11.8)	1.5 (1.5, 1.5)
0.02	8	20,000	380	52.7	4,592.2	87,111	340.8	13.5 (13.4, 13.5)	1.3 (1.3, 1.3)
0.02	4	20,000	380	52.7	6,508.8	61,457	346.5	18.8 (18.7, 18.9)	0.9 (0.9, 0.9)

*, results come from 100 simulations for each pool size, assuming a fixed specificity of 99.5%. Sensitivity for the initial pool size was taken from *Table 2*, and sensitivities for splitting pool sizes were taken as 100% (e.g., in simulations for pools of size 32, the sensitivity for smaller pool sizes were all equal to 100%); †, we took only a third of the relative efficiency because we used pools of RNA, and the amplification phase comprises about a third of the total cost of a RT-qPCR test in our lab. TP, true positive; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; RT-qPCR, real-time quantitative polymerase chain reaction.

Discussion

The average difference in Ct between individual and pooled tests in our study, from 2.21 in pools of size 4 to 3.94 in pools of size 32, were consistent with expected changes of approximately 1 Ct cycle for each twofold dilution of a positive sample (2 to 5 Ct) (30). They were also similar to those found in other studies using the E gene and RNA pools of similar sizes (18,19,31).

Pools of 4, 8, 16, and 32 specimens had sensitivity of 91.1%, 89.6%, 85.8%, and 82.5%, respectively. These estimates were similar, but more precise than those from Gupta *et al.* (95.4%, 95% CI: 77.1%, 99.9%; in pools of 8 samples) (31), Ben-Ami *et al.* (100%, 95% CI: 78.2%, 100%; in pools of 8 samples of nasopharyngeal swaps) (18), and Garg *et al.* (100%, 95% CI: 94.1%, 100%; in pools of ten samples of nasopharyngeal swaps) (32).

The efficiency of pools of eight or more samples was 30% to 280% higher than that of individual testing, depending on the level of prevalence. The relative efficiency of pools of size 32, without discounting costs of specimen collection and RNA extraction, were 11.5, 7.5, and 4.6 for prevalence of 0.5%, 1%, and 2%. These figures were similar to those obtained using de Wolff *et al.*'s simulation approach: 12.6, 8.6, and 3.6, respectively, had they not assumed a doubling of the time to do the pooled tests (29). Efficiency estimates for pools of size 4 were also similar to those from Aragón-Caqueo *et al.* (33). Moreover, pool testing efficiency increases significantly at sensitivity values much lower than those in our study (18,27,29). Indeed, for a sensitivity of 75% and a prevalence of 2%, pools of 32 samples would have a relative efficiency of 4.5, according to our simulations.

In our simulations, we assumed sensitivity was 100% in pools nested within a parent positive pool. If a nested pool were negative, most labs would continue testing the smaller nested pools, because it is safer assuming the parent pool test was a TP than a false positive (FP) (18). Were the parent pool test a FP, further testing in nested pools would identify it as such, because repeated testing in nested pools practically decreases the FP rate to zero (29). This characteristic makes pool testing particularly useful for mass testing of populations with low prevalence of infection. In contrast, individual testing could result in higher numbers of FP than TP, if the FP rate is higher than the prevalence of infection.

Due to its high efficiency and extremely low FP rate, pool testing makes possible not only controlling outbreaks, but also loosening lock-down and social distancing restrictions, by preventing the isolation of FP cases. For a 1% prevalence of infection, using 20,000 RT-qPCR tests in RNA pools of 32 samples would allow discarding SARS-CoV-2 infection in more than 180,000 individuals, who could safely return to work. Moreover, if the main goal is identifying uninfected individuals and returning them to work, pool testing could be used in populations with a high point prevalence of SARS-CoV-2 infection, such as health care workers.

A narrow focus on patient care has prevented the use of pool testing in ML-LIC. This has hampered the detection, tracking, and isolation of asymptomatic cases to stop the transmission of SARS-CoV-2. Even when the fraction of positive tests and the demand for tests have decreased, efforts for mass testing have been limited. Although mass testing initially increases the number of cases, in the long term the strategy leads to lower viral transmission, decreased mortality, and quicker reactivation of the economy (34).

Prevention campaigns based on testing symptomatic and high risk individuals (35), likely have a limited impact on prevention, since asymptomatic infections, which occur in 31% of all cases (36), are responsible for a large fraction of new infections (36). These findings provide strong support for universal instead of selective testing, as a better strategy to prevent new infections, and mitigate health and economic losses (34).

In spite of the abundance of evidence in support of pool testing, from proof of concept (19), statistical (37), simulation (29), and field studies (31,32), recommendations from international health agencies have been non-committal and confusing (17,38). Indeed, it is well established that pool testing could increase cost-efficiency even in populations with a prevalence of infection up to 38% (39), if two-stage Dorfman pooling is used, and up to 10% if binary splitting by halving is used (40). Given the short duration of COVID-19, the prevalence of SARS-CoV-2 infection on the day of testing would be low in the general population, which is the target for mass testing (41,42). In Uruguay, the only LA country using pool testing to prevent transmission (43), the number of tests per case detected (75.2) is 53.4 times higher than in the rest of the region (1.4), and the number of deaths per million is 59 times lower (18.4 *vs.* 1,064.7) (13).

Ideally, the sensitivity of pool testing should be estimated in each lab, to account for differences in methods and experience. However, the Δ and SD_{Δ} from this and other studies could be used to estimate lab-specific dilution-

corrected Ct values and sensitivity of pooled tests in other labs. It is also key for each lab to quantify the additional time required for preparing pools and for positive pools deconvolution to identify positive individuals. Additional time over single testing would depend on each lab specific requirements and resources, poll size used, and prevalence of active infection. This time should be taken into account while calculating the local cost-efficiency of pool testing. There is scarce data on pool testing as compare to individual testing, since this varies significant from lab to lab. However, findings from simulation studies in large populations suggest that pool testing for population-wide screening, such as in health care workers and essential personnel, could be 8–10 times faster than individual testing (29,44). Labs could also use matrix pool testing, i.e., two-dimensional array of rows and columns, with each sample included in a row-pool and a column pool to avoid pool deconvolution (45,46). If both pools defining an intersecting cell in a two-dimensional array were positive, then the shared sample in that cell would be positive, and no additional testing would be needed (29). We have adopted this approach in our lab.

Our estimates of sensitivity are conservative due to the use of a single gene target. Even though the E gene is the most sensitive to detect SARS-CoV-2 (23,47). Moreover, our pooled test sensitivity and efficiency could be underestimated, because our simulation approach did not account for clustering of positive samples in the same pool (42). Another limitation of our study is the use of pools of RNA, which were readily available, instead of pools of nasopharyngeal swaps before RNA extraction. The latter approach saves time and resources dedicated to RNA extraction in single samples. Therefore, our estimates of pool testing efficiency are lower than they would be if pools of nasopharyngeal swaps were used.

In summary, pool testing could increase the number of people tested several times, decrease the cost of testing per TP detected, decrease test result reporting time, and curtail the transmission of SARS-CoV-2, by making possible the identification and isolation of symptomatic and asymptomatic infected individuals.

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Footnote

Reporting Checklist: The authors have completed the STARD reporting checklist. Available at https://jphe.amegroups.com/article/view/10.21037/jphe-21-97/rc

Data Sharing Statement: Available at https://jphe.amegroups.com/article/view/10.21037/jphe-21-97/dss

Peer Review File: Available at https://jphe.amegroups.com/article/view/10.21037/jphe-21-97/prf

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at https://jphe.amegroups.com/article/view/10.21037/jphe-21-97/coif). LEB serves as an unpaid editorial board member of *Journal of Public Health and Emergency* from February 2020 to January 2024. The other authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was approved by the UIS' and the University of Wisconsin-Madison's Institutional Review Boards (IRB00003739). The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). No informed consent was required, since the study was based on respiratory samples stored before the start of our study.

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