Detection of SARS-CoV-2 in Exhaled Breath from Non-Hospitalized COVID-19-Infected Individuals: A Proof-of-Concept Study

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Research Article

Keywords: COVID-19, SARS-CoV-2, oropharyngeal

Posted Date: December 16th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-1066707/v1>

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Abstract

Background: The diagnosis of COVID-19 is based on detection of SARS-CoV-2 in oro-/nasopharyngeal swabs, but because of uncomforness and minor risk for the tested individual during the swab procedure, detection of SARS-CoV-2 has been investigated in other biological matrixes.

Methods: In this proof-of-concept study, confirmed SARS-CoV-2-infected individuals performed a daily air sample through five days. Air samples were obtained through a non-invasive electrostatic air sampler. Detection of SARS-CoV-2 RNA were determined with qRT-PCR. The association of positive samples with clinical characteristics was evaluated through mixed-effect models.

Results: We obtained 665 air samples from 111 included patients with confirmed SARS-CoV-2 infection. Overall, 52 individuals (46.8%) had at least one positive air sample, and 129 (19.4%) air samples were positive for SARS-CoV-2. Patients with symptoms or a symptom duration $\leq$ four days had significantly higher odds of having a positive air sample. Cycle threshold values were significantly lower in samples obtained $\leq$ 4 days from symptom onset. Neither variant of SARS-CoV-2 nor method of air sampling were associated with a positive air sample.

Conclusion: We demonstrate that SARS-CoV-2 is detectable in human breath, and suggest further evaluation of air sampling considering the non-invasive, point-of-care method.

Introduction

The gold standard in the diagnosis of COVID-19 is detection of SARS-CoV-2 genomes from naso- or oropharyngeal (OP) swabs using real-time reverse transcription polymerase chain reaction (RT-PCR). However, the procedure of obtaining material from the upper respiratory tract is invasive and thus uncomfortable and with minor risk for the tested individual. Furthermore, it requires trained staff that risk infection following virus exposure during the swabbing procedure. Therefore, detection of SARS-CoV-2 based on other sample materials have been investigated including plasma, serum, urine, and feces¹. It is well-known that the virus, among other routes, transmits through aerosols and droplets from infected individuals. This has given the rationale of investigating exhaled breath as an alternative specimen type in the diagnosis of COVID-19 as well as a focus on the strategy to mitigate the transmission of SARS-CoV-2²⁻⁴. Air sampling of exhaled breath could provide an easy non-invasive testing alternative with potential for self-sampling. Furthermore, studying the emission of SARS-CoV-2 in exhaled breath of infected individuals can provide valuable information e.g. in relation to identifying high risk exposure activities.

A previous study established that SARS-CoV-2 was detectable in air close to the snouts of minks using an electrostatic air sampler (AeroCollect®, FORCE Technology, Hørsholm, Denmark)⁵. The aim of the current proof-of-concept study was to investigate whether the AeroCollect® device was capable of detecting SARS-CoV-2 in exhaled breath from humans and to investigate if the detection rate differed by duration of symptoms, the variant of SARS-CoV-2 and method of air sampling.

Material And Methods

Study cohorts

This prospective, observational proof-of-concept study (cohort 1) was performed at Amager-Hvidovre Hospital, Copenhagen, Denmark, between December 2020 and May 2021.

Individuals with recently nucleic acid amplification test (NAAT) confirmed SARS-CoV-2 positivity were identified through the Department of Clinical Microbiology. Eligible participants (not hospitalized and ≥ 18 years) and their household members (not hospitalized and ≥ 12 years) were invited to participate in the study. Prior to study entry, all participants provided written informed consent. The study was approved by the Regional Data Protection Centre (record no. P-2020-1219). The study was exempted from ethical approval according to the Regional Ethics Committee of the Capital Region of Denmark (record no. H-20020554). The decision was made because the committee assessed that the aim of the current study was to achieve knowledge about SARS-CoV-2 in the environment and not about human biology. All methods were performed in accordance with the relevant guidelines and regulations.

Oropharyngeal swabs for NAAT was obtained from household members at day one and five. The swabs were analyzed at the Department of Clinical Microbiology and included sequencing of SARS-CoV-2 if the virus was detected. Clinical information at baseline from each participant and information of symptoms of SARS-CoV-2 from day one to five were provided in a questionnaire and registered in the REDCap database.

Cohort 2 consisted of hospitalized individuals with confirmed SARS-CoV-2 infection. These individuals were included in March 2020.

Sample collection

Sampling of aerosolized SARS-CoV-2 was carried out using the AeroCollect® air sampling technology. The AeroCollect® air sampler was mounted with chip-based sample chamber and operated at a flowrate of 0.2 liter per minute. During the sampling, viruses present in the exhaled air were captured and retained electrostatically in the sample chamber. Particle emission rates of aerosols have been shown to be positively correlated with vocal loudness, and thus are higher in singing and loud talking than during normal talking or breathing⁶⁻⁸. Therefore, air samples in this study were obtained from the breathing zone of the participants while singing or talking loudly.

Participants in cohort 1 were requested to provide one air sample per day during five consecutive days. Time of air sampling were four minutes per sample. In order to evaluate air sampling methods, a subset of participants in cohort 1 was asked to provide two matched air samples a day for five consecutive days, one singing/loud talking for four minutes and one exhaling air for four minutes, for evaluating methods on air sampling.

Patients in cohort 2 provided one air sample per patient by exhaling for one minute.

All samples were blind coded and transported within one week of last sampling day to FORCE Technology, Hørsholm, Denmark for analysis.

Detection of SARS-CoV-2 in air samples

Aerosolized SARS-CoV-2 were eluted from the AeroCollect sample chamber using 25 µl nuclease-free water, flushing the chamber 15 times. The Agilent AriaMX Real-time PCR system (Agilent, United States) was used for detection of SARS-CoV-2. The detection was performed in duplicates using the ViroType SARS-COV-2 detection assay, consisting of the Virotype Mix +IC(JOE)-RNA reaction mixture (INDICAL BIOSCIENCE, Germany) and the Virotype SARS-CoV-2 (E-Sarbeco) Primers/Probes mix (INDICAL BIOSCIENCE, Germany), targeting the E gene of SARS-CoV-2. The RT-PCR reaction was carried out according the manufacturer's recommendations using eight µl sample template in a total reaction volume of 25 µl. Reaction conditions were RT at 50°C for 10 minutes, activation at 95°C for two minutes followed by 45 cycles of 95°C for five seconds and 60°C for 30 seconds.

All reactions contained an internal reaction control. In each PCR run, a positive control consisting of the Virotype SARS-CoV-2 (E-Sarbeco) Positive Control (INDICAL BIOSCIENCE, Germany) and a negative template control (nuclease-free water) was included. Fluorescence was measured at the end of each cycle. Baseline corrected raw fluorescence (ΔR) in the Agilent AriaMX software 1.71 (Agilent) was used for setting the fluorescence thresholds. Signals exceeding this threshold with cycle threshold (Ct) value of 45 or below were considered positive.

Interpretation of RT-PCR results of the air samples were blinded to the clinical information of the participants. Analysis results of the air samples were subsequently sent to a clinician at Hvidovre Hospital who combined the data with the clinical information.

Statistical analysis

Baseline characteristics are presented as numbers with percentages or medians with an interquartile range (IQR).

To assess any association of positive air samples between groups of participants, we performed generalized linear mixed-effects models. Fixed effects in the models were symptom duration ($\leq$ four or $>$ four days), symptoms (yes or no), variant of SARS-CoV-2 (wildtype or B.1.1.7) and method of air sampling (singing/loud talking or exhaling air), respectively, corresponding to the subgroups of interest. We used random within person intercept to account for baseline variability between participants in all four models. Furthermore, random intercept of each day of air sampling were included in the model of method of air sampling to account for variability between days.

Difference in Ct values between the abovementioned subgroups were assessed with linear mixed-effects models. Fixed effects in the models corresponded to the subgroup of interest, and random intercept of each participant were also included. Additionally, random intercept of each day of air sampling were included in the model.

Estimates of association were presented as odds ratios (OR) with 95% confidence interval (95% CI). Goodness-of-fit for generalized linear mixed models was evaluated with Hosmer-Lemeshow test.

P values < 0.05 were considered statistically significant. Data analyses were performed using R version 3.6.0 (R Foundation for Statistical Computing, Vienna, Austria).

Results

We recruited 133 individuals of which 22 were excluded (Figure 1) resulting in 111 included subjects with NAAT confirmed SARS-CoV-2. Clinical characteristics of included participants are shown in Table 1.

Table 1
Characteristics of subjects with confirmed SARS-CoV-2 infection.

Characteristics	Overall (n = 111)
Sex	
Female, n (%)	66 (59.5)
Male, n (%)	45 (40.5)
Age, median [IQR]	40 [29, 54]
Comorbidity	
Yes, n (%)	33 (30.0)
No, n (%)	77 (70.0)
SARS-CoV-2 confirmed prior to inclusion	
Yes, n (%)	106 (95.5)
No, n (%)	5 (4.5)
Symptoms at inclusion	
Yes, n (%)	100 (90.1)
No, n (%)	11 (9.9)
Symptom duration at inclusion, median [IQR]	3 [2, 5]
Missing, n (%)	2 (2)
Symptoms at any time	
Yes, n (%)	105 (95.5)
No, n (%)	5 (4.5)
Missing, n (%)	1
SARS-CoV-2 subtype	
Wildtype, n (%)	18 (19.8)
B.1.1.7, n (%)	73 (80.2)
Missing	20
Full air sampling	
Yes, n (%)	102 (91.9)
No, n (%)	9 (8.1)
Abbreviations: n, number; IQR, interquartile range; SARS-CoV-2, severe acute respiratory coronavirus 2.	

Characteristics	Overall (n = 111)
Any positive air sample	
Yes, n (%)	52 (46.8)
No, n (%)	82 (73.9)
Abbreviations: n, number; IQR, interquartile range; SARS-CoV-2, severe acute respiratory coronavirus 2.	

Of the 111 individuals in the study, 52 (46.8%) had at least one positive air sample. In all, we obtained 665 air samples, 536 during singing/talking loud and 129 during exhaling air, from the NAAT confirmed individuals (Table 2). Of the 665 air samples, 129 (19.4%) were found positive for SARS-CoV-2.

Table 2
Number of air samples (positive/negative) stratified by subgroup.

Subgroup	Positive air samples, n (%)	Negative air samples, n (%)	Total air samples, n
All air samples*	129 (19.4)	536 (80.6)	665
Symptoms**			
Symptomatic patients	95 (21.1)	355 (78.9)	450
Asymptomatic patients	6 (7.2)	77 (92.8)	83
Duration of symptoms**			
Symptom duration $\leq$ 4 days	49 (28.0)	126 (72.0)	175
Symptom duration > 4 days	44 (14.5)	260 (85.5)	304
Variant of SARS-CoV-2**			
Wildtype	18 (20.7)	69 (79.3)	87
B.1.1.7.	78 (22.0)	276 (78.0)	354
Method of air sampling			
Singing/loud talking	101 (18.8)	435 (81.2)	536
Exhaling	28 (21.7)	101 (78.3)	129
Abbreviations: n, number; SARS-CoV-2, severe acute respiratory coronavirus 2.			
*obtained through singing/loud talking and exhaling			
**obtained through singing/loud talking			

Subgroup analyses

Figure 2 shows the distribution of air samples (singing/loud talking) obtained according to days from symptom onset. Generalized linear mixed-effects model estimates of having a positive air sample stratified by subgroups are demonstrated in Figure 3. Linear mixed-effect models for Ct values stratified by subgroups are presented in Table 3.

Table 3
Linear mixed-effect model analyses for Ct values stratified by subgroups.

Subgroup	Mean Ct value [95% CI]	Mean difference of Ct value [95% CI]	P value
Symptoms			
Asymptomatic patients	37.17 [34.75; 39.60]	Ref	
Symptomatic patients	35.77 [35.16; 36.38]	-1.40 [-3.90;1.09]	0.267
Duration of symptoms			
Symptom duration > 4 days	36.74 [35.88; 37.59]	Ref	
Symptom duration ≤ 4 days	34.87 [34.08; 35.68]	-1.86 [-3.05;-0.68]	0.002
Variant of SARS-CoV-2			
Wildtype	35.80 [34.43; 37.18]	Ref	
B.1.1.7.	35.71 [35.05; 36.36]	-0.10 [-1.63;1.42]	0.894
Method of air sampling			
Exhaling	35.54 [34.22; 36.86]	Ref	
Singing/loud talking	35.92 [35.02; 36.83]	0.38 [-0.90;1.67]	0.545
Abbreviations: Ct, cycle threshold; CI, confidence interval; IQR, interquartile range; Ref, reference; SARS-CoV-2, severe acute respiratory coronavirus 2.			
*of samples obtained through singing/loud talking			

Individuals with symptoms at time of air sampling had significantly higher odds of having a positive air sample compared to asymptomatic individuals (OR, 4.4; $p = 0.017$). The mean difference of Ct values between symptomatic and asymptomatic patients was not significant ($P = 0.267$).

Odds of having a positive air sample among participants with short duration of symptoms ($\leq$ four days) compared to longer symptom duration ($>$ four days) were significantly higher (OR, 2.2, $p = 0.015$). Cycle threshold values of air samples from participants with shorter duration of symptoms were significantly lower compared to samples obtained from participants with longer symptom duration ($p = 0.002$).

We found no difference in neither OR of having a positive sample nor Ct values between groups of SARS-CoV-2 variant (B.1.1.7 or wildtype) or method of air sampling (singing/loud talking or exhaling), respectively.

When evaluating assumptions of linear mixed-effect models, some models did not have normal distributed residuals. Therefore, secondary models excluding measurements deviating from the normal assumption was fitted for comparison. None of the secondary models let to different interpretation of the results, why results from the original models are presented.

Hospitalized participants

Nine patients hospitalized with COVID-19 were included in March 2020. Days from symptom onset were between seven and 17 at time of air sampling. No air samples from these hospitalized patients were positive.

Discussion

In this proof-of-concept study, we demonstrate that SARS-CoV-2 can be identified in exhaled breath from humans. This is the first study to evaluate SARS-CoV-2 in exhaled breath over five consecutive days in more than 100 confirmed SARS-CoV-2 patients providing more than 650 air samples.

Different studies have investigated exhaled breath as a biological matrix in the diagnosis of SARS-CoV-2⁹⁻¹⁴. The diversity of devices and techniques is wide which is reflected in the varying detection rates (11.1%-70.0%) of these studies. We found an overall detection rate of 19.4% when including all analyzed air samples, which were obtained from one day before through 17 days after symptom onset. The detection rate increased to 28.0% in earlier stages of the disease, i.e. in samples obtained $\leq$ four days from symptom onset. When taking individual variability of each patient into account, the odds of having a positive sample were 2.2 times higher in air samples obtained $\leq$ four days from symptom onset compared to $>$ four days. This corresponds to the significantly lower Ct value observed in these samples compared to Ct values in samples obtained in later stages of the disease. Furthermore, air samples obtained from the second cohort with hospitalized patients with symptoms for at least seven days were all negative. In previous papers, viral load in nose and/or throat swabs has been shown to peak on or soon after the day of symptom onset and then deteriorate, which is in accordance with our results¹⁵⁻¹⁷. Other papers using air sampling for detection of SARS-CoV-2 have also reported higher detection rates and/or lower Ct values with shorter symptom duration^{10,11,14}.

Patients with symptoms at time of air sampling had 4.4 higher odds of having a SARS-CoV-2 positive air sample compared to patients without symptoms. Previous studies evaluating exhaled air samples from COVID-19 patients only report on symptomatic patients why they are not directly comparable with our results. Although a higher proportion of positive samples from symptomatic participants, no difference in Ct value between symptomatic compared to asymptomatic participants were found in our study. Knowledge on viral load in a- and presymptomatic SARS-CoV-2-infected individuals is scarce, but few

studies suggested that viral load in naso-/oropharyngeal swabs from a- or pre-symptomatic individuals did not differ from viral load in symptomatic individuals¹⁸. In our study, only six (7.2%) air samples from asymptomatic individuals turned out positive, providing a wide IQR for Ct value reflecting a too small sample size of this group. Therefore, this result must be interpreted with caution.

A more transmittable lineage of SARS-CoV-2 (Alpha or B.1.1.7) was rapidly spread in December 2020 in the UK and later to other countries^{19,20}. A study reported lower Ct values (i.e. higher viral load) in upper respiratory material from patients infected with B.1.1.7 compared to those infected with previous lineages²¹. We did not find a significant difference in neither OR og positive samples nor Ct value between air samples taken from B.1.1.7-infected individuals compared with wildtype-infected individuals.

The strength of this study is the large sample size and large number of air samples. Furthermore, our findings are in accordance with the literature, reporting higher detection rates in earlier disease stages. Despite the potential observed using air sampling for detection of SARS-CoV-2 in the early phase of the infection, our results suggest that there are still room for optimization of the method, as our overall detection rate was relatively low compared to other studies on air sampling. This could partly be explained by the fact that we performed RT-PCR on the air samples targeting only the E gene. Ryan et al. reported a mean detection rate of SARS-CoV-2 in air samples of 68% using two targets (E and S) which increased to 93.5% when RT-PCR were performed using four gene targets (S/E/N/Orf1ab)⁹. This indicates that analyzing several genes could increase the overall detection rate. Additionally, our detection rate of positive air samples was low with relatively high Ct values compared with oro-/nasopharyngeal swab testing, and the present study do not suggest air sampling as an alternative diagnostic method to standard oro-/nasopharyngeal swab for COVID-19. However, not all virus particles present in cell material from pharynx necessarily aerosolize, why assessing SARS-CoV-2 in exhaled breath could reflect the infectiousness more appropriate than naso-/oropharyngeal swabs. Considering this and the non-invasive method, the potential of detecting the virus in this biological material is worth exploring. With further optimization of air sampling, this method could play a role in population-based monitoring of SARS-CoV-2 as an early warning system.

In conclusion, we demonstrate that SARS-CoV-2 is detectable in humans using the air sampling device AeroCollect® and that odds of having a positive air sample was significantly higher in earlier disease stages, and in symptomatic individuals compared to asymptomatic individuals. Further investigation on air sampling as a method for detecting SARS-CoV-2 and studying emission and transmission is required.

Declarations

Data Availability

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Acknowledgments

We wish to thank Karsten Brandt Andersen from AeroCollect, FORCE Technology, for method development.

Author contributions

Concept and design: C.L., J.S., T.B. and L.K.D.

Acquisition, analysis and interpretation of data: all authors

Drafting the article or revising it critically for important intellectual content: all authors

Final approval of the version to be published: all authors

Funding

This work have been financed by The Danish Agency for Higher Education and Science.

Competing interests

J.S., K.U. and L.K.D. report employment of AeroCollect, FORCE Technology, Hørsholm, Denmark.

All other authors report no potential conflicts.

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Figures

Figure 1

Flow chart of enrollment of patients. Abbreviations: NAAT, nucleic acid amplification test; OP, oropharyngeal; SARS-CoV-2, severe acute respiratory coronavirus 2.

Figure 2

Total number of air samples and percent positive per day from symptom obtained from confirmed COVID-19 patients.

Figure 3

Generalized linear mixed model analyses of having a positive air sample stratified by subgroups presented as OR. Abbreviations: CI, confidence interval; OR, odds ratio; ref, reference.