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PAPER

Exhaled breath condensate (EBC) for SARS-CoV-2 diagnosis still an open debate

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Abstract

The real-time PCR (RT-PCR) on nasopharyngeal swabs (NPS) is the gold standard for the diagnosis of SARS-CoV-2. The exhaled breath condensate (EBC) is used to perform collection of biological fluid condensed in a refrigerated device from deep airways' exhaled air. We aimed to verify the presence of SARS-CoV-2 virus in the EBC from patients with confirmed SARS-CoV-2 infection by RT-PCR, and to determine if the EBC may represent a valid alternative to the NPS. Seventeen consecutive patients admitted to the Emergency Department of the Policlinico were enrolled in the present study with RT-PCR, clinical and radiological evidence of SARS-CoV-2. Within 24 h from the NPS collection the EBC collection was performed on SARS-CoV-2 positive patients. Informed written consent was gathered and the Ethic Committee approved the study. The mean age of patients was 60 years (24–92) and 64.7% (11/17) were male. Patient n.9 and n.17 died. All NPS resulted positive for SARS-CoV-2 at RT-PCR. RT-PCR on EBC resulted negative for all but one patients (patient n.12). In this study we did not find any correlation between positive NPS and the EBC in all but one patients enrolled. Based on these data which greatly differ from previous reports on the topic, this study opens several questions related to small differences in the complex process of EBC collection and how EBC could be really standardized for the diagnosis of SARS-CoV-2 infection. Further studies will be warranted to deepen this topic.

1. Background

The real-time PCR (RT-PCR) on nasopharyngeal swabs (NPS) is the gold standard molecular method for the diagnosis of SARS-CoV-2 infections [1]. Quick diagnosis is warranted to control the continue spreading of the disease. Indeed this may allow immediate care avoiding further potential worsening of clinical symptoms which may result in diverse shades of severity from mild influenza till the onset of severe ARDS during the acute phase and related treatments [2–7]. However, the time-frame between the infection and the onset of respiratory symptoms may be long [1, 2]. Furthermore, there are limitation to the RT-PCR diagnosis in terms of length of time form swab collection to effective results, patient's discomfort during the NPS and false negative results which may occur in up to 15% of cases [2]. Negative NPS results do not rule out infection, and, in patients with a suspicion of SARS-CoV-2 and negative NPS, lower respiratory tract sampling is recommended [8, 9]. Broncho-alveolar lavage and tracheal aspirates are invasive and impractical in most cases of SARS-CoV-2 infection. Therefore, novel options for sampling the lower respiratory tract have been explored. The analysis of the exhaled breath condensate (EBC) is innovative, completely non-invasive and repeatable [11]. The EBC is an easy to perform collection of biological fluid condensed in a refrigerated device from airways' exhaled air. It allows to detect the presence of active

inflammation in the airways of spontaneously breathing and non-invasive ventilated patients [12–14]. Moreover, the EBC may contain small particles, proteins, but also entire or fragmented viruses, bacteria and fungi [15–17]. Previous studies demonstrated the presence of rhinovirus, human respiratory syncytial virus B, flu A and B in the EBC, even though with lower sensitivity compared to the nasal swab [18]. Some studies in the literature have already demonstrated the feasibility to detect the presence of the SARS-CoV-2 virus in the EBC [19–21]. One study compared COVID19 NPS positive vs NPS negative patients showing that EBC may have higher sensitivity than NPS in detecting the presence of SARS-CoV-2 even in some patients with negative NPS.

The aims of the present study were first, to verify the presence of SARS-CoV-2 virus in the EBC from patients with confirmed SARS-CoV-2 infection by RT-PCR, and second, to determine if the detection of SARS-CoV-2 virus in the EBC may represent a valid alternative to the NPS.

2. Material and methods

This was a prospective, single center, observational, proof of concept study. Consecutive patients admitted to the COVID19 Emergency Department (ED) of the Policlinico University Hospital of Bari SARS-CoV-2 pathway (designed to triage only patients with a potential diagnosis of SARS-CoV-2) were enrolled between 20 December 2020 and 15 January 2021. All the enrolled patients had RT-PCR, clinical and radiological evidence of COVID-19. For case definition, the National Institute of Health (NIH) clinical staging of COVID-19 disease was used [22]. The collection of the EBC was performed on patients with a RT-PCR test positive for SARS-CoV-2 within 24 h from the NPS collection. The EBC was usually performed following the standard technique as shown in figure 1 as per current guidelines and usually before the morning round to not interfere with the busy activity of the COVID19 Emergency room [23]. Informed written consent was collected from all patients before performing EBC testing. The study was approved by the Ethic Committee of the Policlinico University Hospital of Bari (number 42440 May 2021). All procedures were carried out in accordance with the Declaration of Helsinki, as revised in 2013, for research involving human subjects.

The researcher responsible for the samples collection explained in details the method of collection to the study participants, and while performing the procedure was wearing full personal protective equipment (PPE) inside the individual room of the COVID-19 ED ward. NPS were collected using UTM viral transport media (FLOQSwabsTM, Copan Italia, Brescia, Italy). EBC samples were then collected using

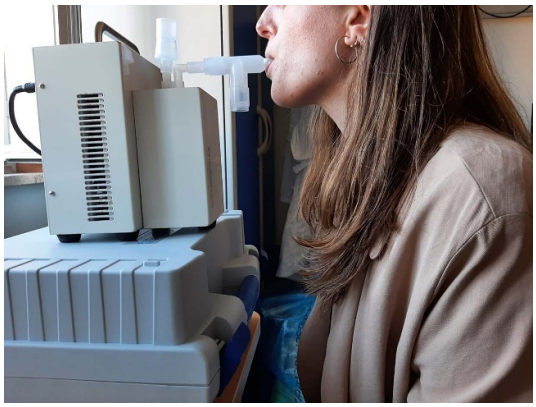

Figure 1.

a Turbo-DECC portable device (Turbo DECCS System, Medivac, Parma, Italy). The mouthpiece was then set up to be connected to a tube that enters the condenser (Turbo DECCS System, Medivac, Parma, Italy) set at the temperature of −5 °C to −10 °C, and attached to a 50 ml falcon conical centrifuge tube. Patients on oxygen mask were asked to switch to oxygen nasal cannulas in order to continue receiving the same amount of oxygen delivered while leaving the mouth free to hold the mouthpiece to collect the EBC material. Once the system was ready for use at the right temperature, the subject was asked to hold the breathing mouthpiece in the mouth and breathe freely usually a variable time from 10 to 15 min or sometimes less depending on patients effort and minimum indispensable time to collect 1.0 ml EBC as showed in figure 1. This was carried out as for the collection standard technique [19–21] and the instruction of the Turbo-DECC portable device. The EBC material was then sampled into Eppendorf test tube and quickly sent to the laboratory of the same hospital. All the EBC were collected and stored at ultra-low temperature (−80 °C) until RT-PCR test.

All samples were processed at the Laboratory of Molecular Epidemiology and Public Health of the Hygiene Unit of the Policlinico Hospital of Bari, which is the Regional Reference Laboratory for surveillance and diagnosis of SARS-CoV-2 infections where NPS were subjected to RT-PCR to detect SARS-CoV-2. While, EBC samples were processed with both RT-PCR and CLEIA (chemiluminescence enzyme immunoassay) antigen tests. The same protocol was used to process NPS and EBC samples RT-PCR. SARS-CoV-2 RNA was extracted from 200 μl of sample using the MagMAX Viral/Pathogen Nucleic Acid Isolation kit (Thermo Fisher Scientific, Waltham, MA, USA) and the KingFisher Duo Prime System (Thermo Fisher Scientific). The extraction protocol envisages an internal control of extraction for each sample. In addition, for each RT-PCR assay, a negative control (RNAase free water) undergoes the

same processing of samples prior to real-time PCR. A three-target commercial multiplex RT-PCR assay that identifies the N, ORF1ab, and S genes was used (TaqPath RT-PCR COVID-19 Assay). The reported sensitivity of the whole process, from RNA extraction to SARS-CoV-2 detection, starting from 400 μl of sample, is 10 genomic copy equivalents for each reaction (400 copies ml^{-1})[21]. Therefore, the sensitivity of the assay on EBC, starting from 200 μl of sample, is 800 copies ml^{-1}. For each RT-PCR assay, 2 μl of positive control (PC) were added. The TaqPath™ COVID-19 PC contains 25 copies μl^{-1}. Results were interpreted through automated software of analysis according to the manufacturer's instructions. For each sample, the cycle threshold (Ct) values were recorded for all three genes.

The EBC samples were also processed via CLEIA antigen test (Lumipulse G SARS-CoV-2 Ag test, Fujirebio, Europe, Ghent, Belgium). It is an assay capable of detecting and quantitatively measuring the presence of SARS-CoV-2 nucleocapsid protein. EBCs were centrifuged at 2000 × g for 10 min and the supernatant was used for the analysis. According to the manufacturer's instructions, the test was considered negative when the antigen level was <1.34 pg ml^{-1} and positive when it was >10 pg ml^{-1}. Values between 1.34 and 10 pg ml^{-1} were considered to be a 'gray zone'.

3. Results

Seventeen patients hospitalized in the Policlinico Hospital of Bari from 1 December 2020 to 15 January 2021 were enrolled. The mean age was 60 years (range: 24–92) and 64.7% (11/17) were male. Clinical severity of the disease at admission of SARS-CoV-2 infection are reported in table 1. Patient n.9 and n.17 died. All the NPS resulted positive for the three targeted genes of SARS-CoV-2 at real time PCR. The Ct values gathered are showed in table 1. The NPS were processed in a single assay. For this RT-PCR assay, the Ct values of the positive control were 27 for the N gene, 28 for the ORF1ab gene and 28 for the S gene, while the Ct value of the negative control was 26.

EBC were collected within 48–72 h from ED admission from all the 17 patients. All the EBC were processed in a single assay. RT-PCR on EBC resulted negative for all but one patients (patient n.12). The Ct values for viral RNA extraction in the EBC positive for SARS-CoV-2 were 33 for the N gene, 33 for the ORF1ab gene and 33 for the S gene. The Ct values reported for the positive control were 28 for the N gene, 29 for the ORF1ab gene and 29 for the S gene, while the Ct value of the negative control was 26. CLEIA antigen test resulted negative for all EBC samples. Clinical characteristics of all patients enrolled are summed up in table 2. All inflammatory markers were elevated and the majority of patients had bilateral lung consolidations at admission. Moreover, all patients were initiated on treatment for SARS-CoV-2 at the admission to the ED as per current guidelines [23].

4. Discussion

The exhaled breath condensate (EBC) contains lower respiratory droplets and is a non-invasive easy to perform sample that may improve the detection of SARS-CoV-2 [20]. In this study we did not find any correlation between the positive RT-PCR diagnosis of SARS-COV-2 at the NPS and the EBC in all but one patients enrolled. Based on the data of the present study, EBC seems to have alternate results which would make it a sampling technique not entirely suitable for the diagnosis of SARS-CoV-2 infection. Indeed, other studies showed that EBC could represent a good alternative to nasopharyngeal swab for the diagnosis [19–21]. All patients admitted to the ED were initiated to standard medical therapy as per current guidelines [23] but it is not proven whether this could affect the viral load in the airways.

The results of the study by Ryan *et al* suggest that testing multiple genes together increases the detection of SARS-CoV-2 [19]. In our study, despite the three targeted RT-PCR, only 1/17 EBC resulted positive for SARS-CoV-2. Therefore, targeting multiple genes in RT-PCR by itself might not improve the detection of the viral RNA in the EBC samples.

A study conducted on the EBC in order to identify a potential route of transmission in healthcare facilities, suggests that the low detection of SARS-CoV-2 in this sample could be related to the reduced viral load of SARS-COV-2 in patients in whom the EBC was collected 27–43 d after symptoms onset [24]. In our study, all patients were enrolled 24–48 h after ED admission with the average onset of respiratory symptoms within five days from admission. Moreover, the EBC collection was performed 24 h after NPS. Although the direct measure of the viral load was not performed, the Ct values did, and they can be considered to be an indirect index of viral load in different specimens and a valuable proxy for infectious virus [25, 26]. The reported Ct values for NPSs collected from our patients were low, and, therefore, we could speculate that almost all of them had a high viral load. It can be postulated that in these patients the viral load was still higher in the upper airways and lower or undetectable as yet in the lower airways via EBC. Indeed, from one side, in previous studies the NPS to EBC interval of patients with positive concordance between NPS and EBC was between 1 and 19 d, thus allowing more time for adequate viral load to be detectable in the lower airways. While, on the other side, those patients with a negative correlation between positive NPS and negative EBS had shorter NPS-EBC time interval of sampling (0–7 d) in line with our results [19]. Interestingly, all patients enrolled in this study

Table 1. Demographic and clinical characteristics of the SARS-CoV-2 positive patients at admission.

Patient number	Age	Sex	Days from symptoms onset	COVID-19 severity	Real time PCR on NPS (Ct value for N, ORF1ab and S genes)	Real time PCR on EBC (Ct value for N, ORF1ab and S genes)	CLEIA Antigen test on EBC
1	73	F	7	Moderate	Positive (28,28,28)	Negative	Negative
2	43	M	12	Moderate	Positive (37,36,36)	Negative	Negative
3	49	M	1	Moderate	Positive (34,34,34)	Negative	Negative
4	82	M	15	Moderate	Positive (33,35,35)	Negative	Negative
5	56	F	2	Severe	Positive (33,33,33)	Negative	Negative
6	75	F	1	Moderate	Positive (9,8,9)	Negative	Negative
7	56	F	3	Moderate	Positive (22,22,23)	Negative	Negative
8	66	M	14	Moderate	Positive (29,29,30)	Negative	Negative
9	92	M	4	Critical	Positive (11,11,12)	Negative	Negative
10	53	M	6	Mild	Positive (24,25,24)	Negative	Negative
11	84	F	1	Moderate	Positive (15,15,13)	Negative	Negative
12	62	F	1	Moderate	Positive (17,17,17)	Positive (33,33,34)	Negative
13	40	M	1	Mild	Positive (32,31,32)	Negative	Negative
14	24	M	7	Severe	Positive (25,25,27)	Negative	Negative
15	35	M	1	Mild	Positive (28,28,29)	Negative	Negative
16	57	M	4	Critical	Positive (23,23,23)	Negative	Negative
17	75	M	4	Critical	Positive (25,23,26)	Negative	Negative

NPS = nasopharyngeal swab.

Ct = Cycle threshold, Double or Single lungs consolidations involvement.

Table 2. Clinical characteristics of the SARS-CoV-2 positive patients at admission.

Clinical characteristics	Mean (SD)
Ferritin	449.7 (396.1)
LDH	319 (187)
D-Dimer	835.2 (963.3)
PCR	74 (46)
WCC tot	11 113 (9746.2)
Lymphocytes	1235.4 (938.5)
Chest x-ray	D/S 15/2

WCC = White cell count.
D = Double; S = Single lung consolidations involvement.

had symptoms with a variable range from mild to critical disease. Therefore, it would have expected the viral load to be already present and highly represented in the lower airways too. Especially because of the presence of bilateral lung consolidations in the majority of patients enrolled as per table 2. In spite of this, SARS-CoV-2 was detected only in 1/17 EBC sample. Another hypothesis for the lower detection of the viral load into the BEC samples could be related to the transfer the virus requires from the upper to the lower airways. In the latter initially, it may get diluted into the larger lower airways surface therefore it may take time to sufficiently replicate, to produce alveolar/capillary barrier disruption and/or lung consolidations and to become detectable in the EBC sampling. Other studies have demonstrated similar negative results in detecting Influenza virus in the EBC samples with very low outcomes [27, 28]. Third, the timing used in this study for the EBC collection could have been an important justifying factor for the low viral load. Indeed, in the literature no preferred timeframe is suggested for EBC collection. The morning time chosen for the EBC collection in this study was in accordance with the personnel working in the Emergency department to avoid interfering with the normal routine activities of the busy COVID19 ED. However, we could hypothesize that a different time may have influenced the amount of samples collected and therefore the higher or lower viral load collected. Furthermore, it is also possible that at some point in the infection curve when the viral shedding is completed by the patient, this may have reduced the virus-rich sampling. However, the patients enrolled were all recently admitted in the ED so with very short timing between the infection and actual symptoms manifestation. Potential other problems that can be encountered are that Turbo-Deccs collects at low temperature set-up at −10 C which may be sub-optimal for collecting the aerosol fraction of EBC viral particles that are best collected at −80 C. Moreover, saliva may be collected and it may dilute the amount of EBC collected altering the detection of SARS-CoV-2 [10, 29–31]. This study has some limitations. First, the small number of patients enrolled. Increasing the sample size may have resulted in different outcomes. However, given the clear difference with previous studies and the expensive techniques adopted, the study was interrupted for negative results. Second, a direct measure of the viral load was not measured in these samples, however, Ct values were considered instead, and, as other studies confirmed, given the valuable proxy for infectious virus detection, Ct values can be considered as accurate as viral load given the close correlation between Ct value/viral load and cultivable virus. Third, the single positive result could be affected by the procedures for samples processing since the study lacks laboratory tests aimed at assessing the recovery rate of the viral RNA with the device. Furthermore, since the SARS-CoV-2 detection method was very sensitive [20], the results could be affected by the method of EBC collection as no clear clinical or laboratoristic difference was noted in the single positive sample collected compared to the others. Further studies are necessary to address this aspect. Fourth, the timing between NPS and EBC may be too short to find high viral concentration in the lower airways. However, no studies have compared the correct timing from symptoms onset and EBC sampling in patients with SARS-CoV-2 neither in previous other viral illness infection. The strength of this study is that given the negative results and the great difference with previous studies present in the literature opens several questions related to the correct timing, amount of EBC collected, and type of machine used for EBC sampling in patients with SARS-CoV-2 infection. Small differences in the complex process of EBC collection may greatly alter the outcomes and may contribute to invalidate results and efforts of patients and health care providers involved. Therefore, further studies will be warranted to deepen this topic.

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Declaration of interest

All the authors declare no competing interests with the present study.

Authors contribution

All authors made substantial contributions to the conception and design of the study, acquisition of data, analysis and interpretation of data, drafting the article, revising it critically for important intellectual content, and final approval of the version to be submitted.

Data availability statement

The data that support the findings of this study are available upon reasonable request from the authors.

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