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TITLE:

Rapid Non-invasive Screening of COVID-19 by Analysing Volatile Organic Compounds in Exhaled Breath: COVID-19 Breathalyzer Study

CLINICAL TRIAL NUMBER: DSREC-01/2021_07

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1. Introduction

The ongoing COVID-19 pandemic is due to infection caused by the novel SARS-CoV-2 virus. The number of confirmed cases globally has exceeded seventy-five million and the impact of the pandemic on healthcare, social and economic structures across the world is unprecedented. The massive roll out of screening tests to identify infected individuals and ensure appropriate implementation of isolation measures and tracing of their contacts has been identified as a crucial element for success in containing the spread of SARS-COV-2. The viral entry into cells is via ACE2 receptors with the nasopharynx being one of the key sites for initiation of the infection. Therefore, nasopharyngeal swab (NPS) has emerged as the gold standard respiratory specimen with the RT-PCR being the main laboratory diagnostic approach for testing. However, NPS-based molecular testing with RT-PCR requires the invasive collection of a NPS, often associated with discomfort to the person being tested and potentially exposing the healthcare worker collecting the sample because of close contact. The typical need for trained healthcare workers to collect NPS and use of personal protective equipment during collection has put additional strain on healthcare. Alternative specimens for molecular testing such as saliva has addressed some of these obstacles. However, there remains a need for faster, accurate and more scalable solutions for population surveillance.

Analysis of volatile organic compounds (VOCs) in exhaled breath is an attractive technology for affordable, rapid, accurate and non-invasive testing for COVID-19. Using a mass spectrometer, molecular characterization of hundreds of VOCs in exhaled breath may be used to generate a unique bio-fingerprint for COVID-19. The technology is easy to use and is completely

non-invasive as the person needs to only exhale into a disposable mouthpiece connected to the system.

Building on early studies, we aim to validate the use of VOCs in exhaled breath in the Breathonix breathalyzer platform as an alternative to molecular based COVID-19 testing with nasopharyngeal swabs in a larger and more diverse ambulatory population in Dubai, UAE. Validation of such rapid and non-invasive point-of-care screening methods are critical as countries begin to ease restriction measures and economies begin to recover.

2. Objective:

To validate the use of Breathonix's breathalyzer technology of analysing VOCs in exhaled breath as an alternative to molecular-based RT-PCR testing, using nasopharyngeal swabs, for COVID-19.

Specific aim 1: To estimate the (1) sensitivity, (2) specificity, (3) accuracy, (4) positive predictive value, and (5) negative predictive value of using breathalyzer method with reference to NPS-based molecular RT-PCR as standard.

Specific aim 2: To estimate the correlation of test results from the analysis of VOCs in breath obtained from exhaling into (1) a disposable mouthpiece connected to the MS system with real-time analysis as compared to (2) a custom designed breath bag with offline analysis.

3. Methods:

Participants and consent: Any individual presented to the designated study

site for COVID-19 testing, who is able to (1) independently exhale into the mouthpiece and/or breath bag and (2) provide informed consent will be eligible for this study. Verbal consent for participation in this study will be obtained using a preset script which explains the purpose, voluntary nature, and anticipated benefits and harms of the study. The script will read as follows:

“We are conducting a study to see if a breath test is as good as a nasopharyngeal swab in testing for COVID-19. Your participation is completely voluntary, and your care will not be affected if you chose not to participate in it. There is no fee or reward for your participation. If you agree to participate, we will only ask you to breath into a machine. And in some cases, we may also ask you to breath into a bag. Everything that you will be touching in this breathing test is for single-use only (in another words, you will be the only person breathing into the mouthpiece or the bag). This test should not take more than a minute. To ensure that the results are accurate, we will also be testing the nasopharyngeal swab sample you have provided for other bacteria or virus in your respiratory tract Some simple information (collected as part of your registration today) will also be collected and used for this study. Full confidentiality will be maintained at all times and your personal identity is protected. Your participation will help advance our knowledge on the best method to test for COVID-19 and will support efforts to develop faster and more convenient testing programs. Thank you for your willingness to participate in this study.”

Setting: The study will be conducted in the Nad Al Hamar ambulatory health clinic run by the Dubai Health Authority. This site was chosen given recent historical data of COVID-19 testing volumes and positivity rates as well as logistical considerations of facility size to accommodate the mass spectrometer.

NPS collection and RT-PCR testing: NPS specimens will be obtained by trained healthcare personnel using standardized DHA NPS collection protocol for COVID-19 screening. The NPS will be placed in a Greiner Bio-One universal transport system and transported to the central DHA Virology Laboratory for processing. NP samples will be processed using validated RNA extraction and SARS-CoV-2 RT-PCR protocols used in the DHA virology laboratory. Viral RNA will be extracted from 200 µL of each sample using the EZ1 DSP Virus Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The internal control (10 µL), which is composed of MS2 phage genome for validation of the RNA extraction and reverse transcription will be added before extraction. SARS-CoV-2 RT-PCR for the detection of three gene targets (*N*, *E* and *RdRp* genes) will be carried out using the Allplex™ 2019-nCoV Assay (Seegene, Seoul, South Korea) in accordance with manufacturer provided instructions. Purified nucleic acid will be reverse transcribed using 5X Real-time One-step Buffer/Real-time One-step Enzyme into cDNA which will then be subsequently amplified in a CFX96 Real-Time PCR Detection System (Biorad, California, USA). Cycle threshold of ≤40 will be taken as cut-off for positive result for the target genes as per manufacturer provided protocol. The Seegene Viewer Software V3.20 will be used for analysis and interpretation of the RT-PCR results. A presumptive positive result will be rendered if only the *E* gene target is detected, and a

positive result will be based on the detection of any two target genes. A negative result will be reported if no gene targets are amplified, and the internal controls are validated. To rule out any concurrent infection with other respiratory pathogens, the NPS sample will also be tested using the Biofire Film Array multiplex PCR platform using both the pneumonia and respiratory panels (bioMérieux, Marcy l'Etoile, France). The Biofire Filmarray Pneumonia panel detects 27 bacteria and viruses while the Respiratory panel tests for 19 viruses and 4 bacteria that cause respiratory tract infections. The full list of the pathogens detected in each panel is shown in Appendix. The Biofire Filmarray is an automated multiplex PCR platform which integrates sample preparation, nucleic acid extraction and purification, amplification, detection, and analysis into one simple system with 2 minutes of hands-on time, and a total run time of about one hour.

Breath testing: Participants will be asked to either directly breath into a disposable single use mouthpiece connected to the mass spectrometer for real-time analysis (n=1,000) or into a custom designed breath bag for offline analysis (n=800). This will yield a total of 1,800 participants with both a NPS for RT-PCR and a breath test and will address **Aim 1** of the study (**Figure 2**). Of the participants who will be directly breathing into the mouthpiece (n=1,000), a subgroup (n=200) will also be asked to breath into a custom designed breath bag for offline analysis. This will yield 200 participants with a pair of breath tests ((1) direct breath into a mouthpiece connected to mass spectrometer for real-time analysis and (2) through a breath bag for offline analysis). This will address **Aim 2** of the study (**Figure**). The Breathonix system is designed to ensure user safety and prevent potential cross-contamination. Every participant undergoing the test will use a disposable

single-use mouthpiece with a one-way valve and a saliva trap to prevent (1) accidental inhalation of previous participant's breath and (2) saliva from entering the Breathonix breathalyzer platform. The internal surfaces of the breath sampler and the mass spectrometer that come in contact with the participant's breath samples are operating at 70°C and more than 100°C respectively. Research has shown that the SARS-CoV-2 virus is not able to survive for more than 3 minutes on surface above 70°C [4]. In addition, in between each participant, the external surfaces that the participant may be in contact will be wiped down using alcohol wipe (training will be provided to the study team), making cross-contamination unlikely.

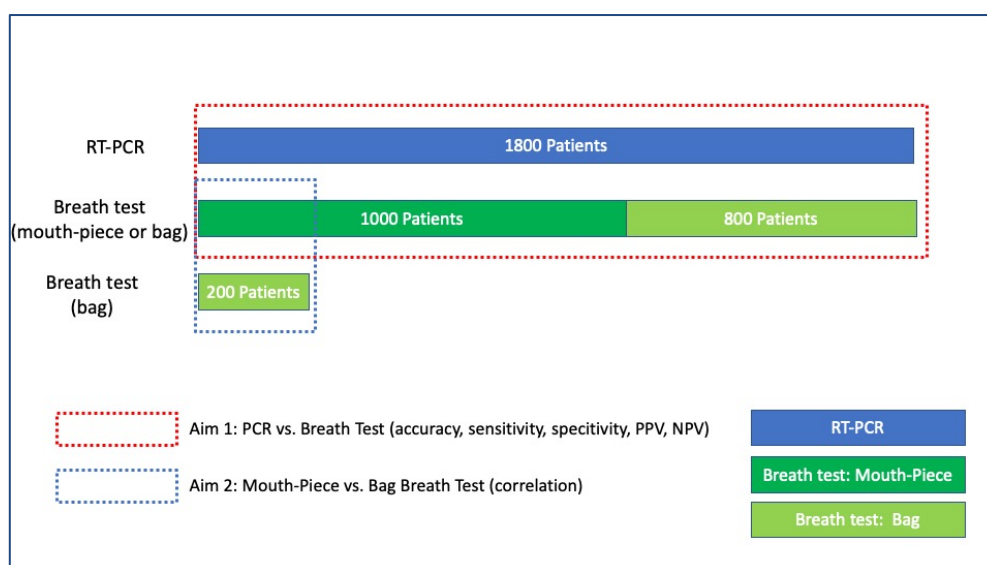


Figure 2. Design of the clinical trial.

The following specific instruction will be provided to the participants to ask them to **directly breath into the disposable single use mouthpiece** connected to the mass spectrometer:

1. Tear open the individually wrapped mouthpiece

2. Inset the mouthpiece to the tubing inside breath sampler



3. Perform one full exhalation with mouth fully covering the mouthpiece
4. Remove the mouthpiece after exhalation and dispose it to 'contaminated waste bin' or equivalent

The following specific instruction will be provided to the participants to ask them to **breath into the breath bag**:

1. Remove the black cap on tedlar bag (if any)
2. Twist the valve handle to an open position (See pic below).
3. Exhale into the bag until it is fully inflated and then close the valve.



4. Check if the valve is fully closed (horizontal position).



Empty Bag



Fully Inflated Bag



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Data Collection: The following data variable will be collected from all study participants:

Participant study ID	
Date of COVID-19 screening	
Indication for COVID-19 screening	Contact with confirmed case / Occupational exposure / Travel
Demographic data/social data	Nationality Age Gender Residency Status /Tourist Travel History (travel dates and country) Current smoking (Y/N) (if yes, pack-year of smoking) Past smoking (Y/N) (if yes, pack-year of smoking)
Healthcare workers only	Working in a COVID-19 ward / isolation center (Y/N) If yes: please state duration; Setting of work: ICU / General ward/Isolation Center / Other
Medical history	Chronic kidney disease Chronic liver disease Chronic neurological disorder Diabetes Hypertension Heart disease Malignant neoplasm Pregnant (if yes; state gestational age in weeks)
COVID-19 symptoms	Yes/NO (if yes, specify symptoms)

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(For each symptom present, the date of onset will be noted)

History of self-reported feverishness or measured fever of $\geq 38^{\circ}\text{C}$
 Cough
 Sore throat
 Shortness of breath
 Myalgia / arthralgia
 Eye pain
 Loss of sense of smell
 Loss of sense of taste
 Other

Vital signs

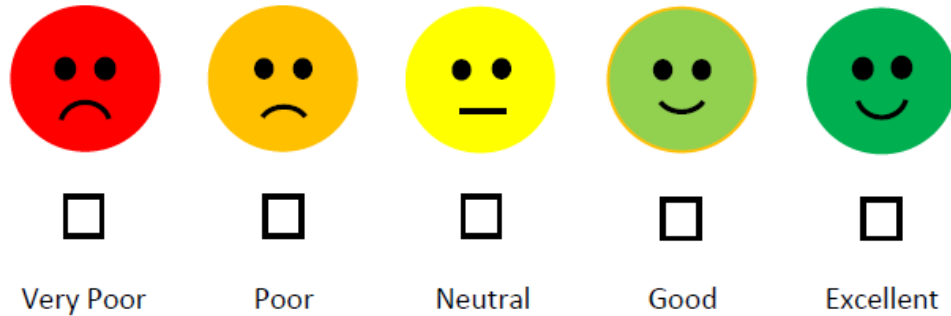
Temperature
 BP
 Oxygen saturation (where taken)
 Height / Weight

Time of last meal, drink and smoke (if applicable)

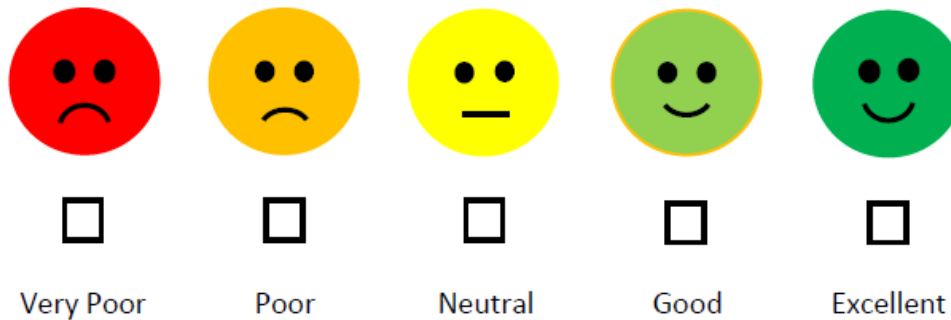
**most of these are information routinely collected from patients presenting for screening*

We will also assess participant's satisfaction level in the breath test experience, as follows:

1 - What was your experience with the Breath Bag? (only for participants who did the breath bag test)



2 - What was your experience with the disposable mouthpiece?



Statistical methods and sample size considerations: Based on an expected infection prevalence of ~10%, we aim for a sample size of 1,800 participants. The estimated infection rate is based on recent trends in the designated clinical site. We will strive to exceed that prevalence in our sample by preferentially inviting subjects with higher pre-test probability for testing positive based on symptoms of a flu-like illness or recent history of high-risk exposure to a person with confirmed COVID-19. This will afford us adequate power of at least 80% to detect a sensitivity of at least 80% and a specificity of at least 95% with an alpha error of 0.05. Categorical variables will be summarized as number (percentages) and continuous variables will be

summarized as mean \pm standard deviation (SD) or median (interquartile range; IQR). Using NPS RT-PCR as the reference standard, the accuracy, sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) for breath tests will be calculated with their associated 95% confidence limits (**Aim 1**, n = 1,800 participants). Kappa coefficient will be used to estimate the agreement between NPS RT-PCR and breath test results. In addition, Kappa coefficient will be used to estimate the agreement between direct real-time mouthpiece breath test and offline breath bag test results (**Aim 2**, n = 200 participants). We will also conduct exploratory analyses comparing the performance of real-time mouth-piece breath testing to offline breath bag testing, using RT-PCR as a reference for both (sample size: 1,000 pairs of RT-PCR and direct mouth-piece breath tests, and 1,000 pairs of RT-PCR and offline breath bag tests, Figure 2). All analyses were carried out using SPSS statistical software version 24 (Chicago, Illinois, USA) and statistical significance was set at p value < 0.05.

Interim Analysis: Once we have a discovery set of 100 positive cases and minimum of 100 negative cases, an interim analysis will be conducted on the correlation of breath VOC profiles and COVID-19 status. The rest of the participants enrolled will be treated as a validation cohort. Algorithm developed (biomarkers identified and cut-off values determined) will be used for predicting the validation cohort.

Data Transfer and Blinding: Breath VOC data labelled with participant study ID will be transferred to Breathonix data analysis team using file sharing in TeamViewer. Clinical Data will be anonymized (patient identifiers replaced with participant study ID) by the MBRU study team and shared to Breathonix

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team via email using password protected excel table. Data from discovery set, particularly COVID-19 positive or negative will be shared to Breathonix team to develop the algorithm. PCR test results of validation set will be blinded to Breathonix team. Breathonix team will predict COVID-19 results based on the algorithm developed and share the results with MBRU team. MBRU team will compare the breath test results with RT-PCT results and calculate the sensitivity and specificity independently.

Significance of study: The findings from this study will provide important information on the utility of analysing VOCs in exhaled breath to screen for COVID-19 using a direct mouthpiece or a custom designed breath bag. If proven to be a valid screening test, the system could potentially be used for rapid and cost-effective mass screening in areas of high human traffic including airports and large conventions.

Research Team

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