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ANALYSIS OF VOLATILE ORGANIC COMPOUNDS IN EXHALED BREATH OF COVID-19 PATIENTS

Tiar Oktavian Effendi[1*], Iin Noor Chozin[1], Suryanti Dwi Pratiwi[1], Arinto Yudi Ponco Wardoyo[2], Nanik Setijowati[3]

[1]Department of Pulmonology and Respiratory Medicine Medical Faculty of Universitas Brawijaya, Dr. Saiful Anwar General Hospital, Malang, Indonesia

[2]Department of Physics, Faculty of Science Universitas Brawijaya, Malang, Indonesia

[3]Department of Public Health, Medical Faculty of Universitas Brawijaya, Malang, Indonesia

*Corresponding Author : email tiar.oktavian@gmail.com +6281332772380

Background: More than 2 years since COVID-19's first cases were reported in 2019. Diagnosis of COVID-19 is a key to controlling the pandemic. Sample for COVID-19 testing is collected by naso-oro-pharyngeal swab. This procedure is often uncomfortable and requires a trained examiner. Exhaled breath contains thousands of *volatile organic compounds* (VOC) which are likely to change during infection.

Aims and objectives: This study aims to analyze the difference of VOC in the exhaled breath between COVID-19 and healthy subjects.

Methods: A cross-sectional study carried out recruiting 90 confirmed cases of COVID-19 and 42 healthy subjects. A sample of exhaled breath was collected by using a 500 ml airbag in both groups. Sample was analyzed using an arrayed sensor breath analyzer to quantify the concentration of CO_2, C_7H_8, C_6H_{14}, CH_2O, NH_4, TVOC, NO_2, PM1.0, CO, NH_3 and Acetone.

Results:

The median of CO_2, NH_4, TVOC, NO_2, and Acetone are significantly lower in COVID-19 patients compared to healthy subjects (respectively 607.3 vs 1175.1; 0.0 vs 1.05; 0.05 vs 146.6; 0.04 vs 1.55; 0.0 vs 0.23) while C_7H_8, CH_2O, CO, and NH_3 are significantly higher (respectively 0.92 vs 0.0; 0.55 vs 0.01; 0.24 vs 0.0; 1.99 vs 0.67; all with p-value of <0.05.). Furthermore, we found NH_4, Acetone, NH_3, and CO are positively correlate with severity of COVID-19, while CO_2 and TVOC are negatively correlate.

Conclusions:

COVID-19 patients emit distinctive VOC profiles in comparison with healthy subjects, and it is related to the severity of the disease.

Key words: Volatile organic compounds, COVID-19, Diagnosis of COVID-19

ANALISIS DARI *VOLATILE ORGANIC COMPOUNDS* PADA UDARA EKSPIRASI PENDERITA COVID-19

Tiar Oktavian Effendi[1*], Iin Noor Chozin[1], Suryanti Dwi Pratiwi[1], Arinto Yudi Ponco Wardoyo[2], Nanik Setijowati[3]

[1]Departemen Pulmonologi dan Kedokteran Respirasi, Fakultas Kedokteran Universitas Brawijaya, RSUD Saiful Anwar, Malang, Indonesia

[2]Departemen Fisika, Fakultas Matematika dan Ilmu Pengetahuan Alam Universitas Brawijaya, Malang, Indonesia

[3]Departemen Ilmu Kesehatan Masyarakat, Fakultas Kedokteran Universitas Brawijaya, Malang, Indonesia

*Corresponding Author : email tiar.oktavian@gmail.com +6281332772380

Latar Belakang: Lebih dari 2 tahun sejak pertama kali kasus COVID-19 dilaporkan di tahun 2019. Diagnosis dari COVID-19 merupakan kunci utama dalam mengontrol pandemi yang terjadi. Sampel yang digunakan untuk pemeriksaan COVID-19 umumnya diambil dari prosedur swab naso-oro-faring. Prosedur ini seringkali menimbulkan rasa tidak nyaman dan memerlukan pemeriksan yang terlatih. Udara ekspirasi pernapasan mengandung ribuan volatile organic compounds (VOC) yang mungkin dapat berubah selama proses infeksi.

Tujuan: Penelitian ini bertujuan untuk menganalisa perbedaan kadar VOC pada udara pernapasan penderita COVID-19 dengan kelompok kontrol, serta menganalisa hubunngan kadar VOC dengan derajat keparahan COVID-19.

Metode: Penelitian *cross-sectional* dilakukan dengan total 90 subjek terkonfirmasi COVID-19 dan 42 subjek kontrol. Sampel udara pernapasan ditampung dalam 500 ml kantong udara pada kedua kelompok. Sampel kemudian dianalisa menggunakan *breath analyzer* untuk menilai konsentrasi dari kadar CO_2, C_7H_8, C_6H_{14}, CH_2O, NH_4, TVOC, NO_2, PM1.0, CO, NH_3 dan Acetone.

Hasil: Nilai median dari CO_2, NH_4, TVOC, NO_2, dan Acetone ditemukan secara signifikan lebih rendah pada kelompok COVID-19 (secara berurutan 607,3 vs 1175,1; 0,0 vs 1,05; 0,05 vs 146,6; 0,04 vs 1,55; 0,0 vs 0,23) sedangkan C_7H_8, CH_2O, CO, dan NH_3 secara signifikan lebih tinggi (secara berurutan 0,92 vs 0,0; 0,55 vs 0,01; 0,24 vs 0,0; 1,99 vs 0,67). Analisa lebih lanjut menunjukkan kadar NH_4, Acetone, NH_3, dan CO memiliki korelasi positif dengan derajat keparahan COVID-19, sedangkan CO_2 dan TVOC berkorelasi negatif dengan derajat keparahan penyakit.

Kesimpulan: Kadar VOC yang ditemukan pada penderita COVID-19 berbeda dengan kelompok kontrol, dan berkorelasi dengan derajat keparahan penyakit.

Kata kunci: *Volatile organic compounds*, COVID-19, Diagnosa COVID-19

INTRODUCTION

Corona-virus disease 2019 (COVID-19) is an infectious disease caused by Severe Acute Respiratory Syndrome Coronavirus-2 (SARS CoV-2). This virus is the third one of the corona family that causes epidemic and continually become pandemic[1]. Until now, it's already infected more than 600 million people in the world and caused more than 6 million deaths[2]. Back on 31th December 2019, World Health Organization (WHO) China Country Office reported several cases of pneumonia with unknown causes, later identified as covid-19 cases[3].

Rapid diagnosis is one of the key means to control the pandemic situation. Standard confirmation of acute SARS-CoV-2 infection is based on the detection of unique viral sequences by nucleic acid amplification test (NAATs), such as reverse-transcription polymerase chain reaction (RT-PCR)[4]. The procedure is not widely available and somehow challenging in many countries. Therefore, developing a less expensive, but faster and reliable enough to diagnose COVID-19 is needed. Rapid diagnostic test or RDT, which is an antigen-detection diagnostic test designed to directly detect SARS-CoV-2 proteins was developed widely[5].

Both of the procedures are testing the sample obtained through respiratory specimens. Testing combined nasopharyngeal and oropharyngeal swabs from one individual has been shown to increase sensitivity and improve reliability[4]. Although obtaining the sample is generally considered safe, it requires semi-skilled staff, often uncomfortable, and several complications have been reported with the possibility of a larger number escaping systemic recording and reporting to date. Several complications of pharyngeal swabs are including a break of the swab's tip, foreign body sensation, epistaxis, dislocation of temporomandibular joint, and leak of the cerebrospinal fluid[6]. Therefore, even only reported in a small number, increase awareness of the complication, and moreover, inventing a new testing procedure with fewer complications but reliable, are needed.

Volatile organic compounds (VOCs) are organic compounds that evaporate easily at room temperature. VOCs can be derived from the environment (exogenous), taken through inhalation or ingestion, and produced within the body[7]. Recent studies have shown at least 1765 VOCs can be detected in humans. Physiological metabolism, product of metabolic processes from microbial pathogens, and host response to pathological processes such as inflammation and infection can affect the VOCs. Thus, VOCs emanating from exhaled breath may provide a deep insight into various biochemical processes in the human body[7,8]. Previous studies have shown bacterial pneumonia, reactive oxygen species (ROS), inflammation, septic condition,

use of a ventilator, and viral infection can emit different VOC profile[7,9]. Infection of SARS-CoV-2 is also believed to have distinguished VOC profile. Analysis of VOCs in the exhaled breath has the potentiality to become a diagnostic test which is not only quick, non-invasive, reliable and available widely[7].

MATERIAL AND METHODS

Study design and participants

An exploratory study, case-control selection, cross-sectional study or two-gate design was conducted in Saiful Anwar General Hospital, East Java Province, Indonesia, and Idjen Boulevard Field Hospital, Malang, Indonesia. Confirmed cases of COVID-19 patients, regardless of their severity, who were admitted to one of these hospitals were randomly selected and provided informed consent. Subjects with acute deterioration, using invasive ventilation, or unable to provide exhaled breath samples were excluded. Healthy subjects were also recruited (no respiratory symptoms with negative results of RDT or RT-PCR for SARS-CoV-2) as a comparison. Subjects in the COVID-19 group were further divided into subgroups based on the severity of the disease.

The severity of the disease is classified based on the national guideline for COVID-19 management in Indonesia. Those without any symptoms were classified as asymptomatic. Mild degree defined as a patient with symptoms such as cough, fatigue, fever, anorexia, shortness of breath, myalgia, sore throat, nasal congestion, headache, diarrhea, nausea and vomiting, anosmia, ageusia, without any sign of pneumonia. Moderate degree is marked by pneumonia with room air saturation equal to or above 93%. If the saturation drops below 93%, then it is classified as the severe degree. The critical degree is defined as those with acute respiratory distress syndrome (ARDS) or septic shock, or sepsis.

Exhaled breath sample collection and data analysis

Subjects in both groups were asked to exhale into 500 ml of a sealed airbag. The valve of the airbag is then opened and connected by a tube to the breath analyzer device. This device is equipped with an arrayed sensor to detect the concentration of CO_2, C_7H_8, CH_2O, NH_4, TVOC, NO_2, NH_3, CO, and Acetone. Results were recorded in a customed program and quantified for further analysis.

Statistical analysis

All data analyses were performed using SPSS TM statistical software package. Comparisons of the concentration of VOCs within COVID-19 and control groups were done using Mann Whitney and unpaired T-Test. Further comparisons in COVID-19's group based on its severity was analyzed using the one-way ANOVA and Kruskal Wallis statistical test. The correlation of the VOCs and the severity of COVID-19 were analyzed using the Spearman test.

RESULTS

A total of 132 participants was included, divided into two groups. Group 1 is defined as the COVID-19 group with 90 confirmed cases of COVID-19 patients. The second group consisted

of 42 healthy subjects, defined as the Control group.

Table 1. Characteristics of subjects

Characteristics		Group 1 (n= 90)	Group 2 (n= 42)	p value
Gender	Male	61 (67.8%)	23 (54.8%)	0.148
	Female	29 (32.2%)	19 (45.2%)	
Age	18-29	27 (30%)	18 (42.9%)	<0.001*
	30-39	9 (10%)	24 (57.1%)	
	40-49	11 (12.2%)	0 (0%)	
	>49	43 (47.8%)	0 (0%)	
Smoking Status	Never smoker	55 (61.1%)	39 (92.8%)	<0.001*
	Ex- smoker	28 (31.1%)	1 (2.4%)	
	Active smoker	7 (7.8%)	2 (4.8%)	
Comorbid	Diabetes Mellitus	14 (15.5%)	1 (2.4%)	0.026*
	CVD	20 (22.2%)	3 (7.1%)	0.033*
	Asthma	4 (4.4%)	6 (14.3%)	0.047*
	COPD	0 (0%)	0 (0%)	-
	Active TB	0 (0%)	1 (2.4%)	0.142
	Obesity	11 (12.2%)	6 (14.3%)	0.742
	Malignancies	0 (0%)	0 (0%)	-
	None	55 (61.1%)	27 (64.3%)	0.726

Definition of abbreviations: CVD = Cardio vascular disease; COPD = Chronic obstructive pulmonary disease; TB = Tuberculosis

Group 1 was further divided based on its severity, 12 (13,3%) subjects were asymptomatic, 33 (36,7%) subjects with mild disease, 10 (11,1%) subjects with moderate disease, 23 (25,6%) subjects with severe disease, 12 (13,3%) subjects were critically ill.

Nine parameters of VOCs from exhaled breath samples were compared between group 1 and group 2. In COVID-19 groups, we found a significantly higher concentration of C_7H_8, CH_2O, CO, and NH_3 when compared to the control group (respectively 0.92 vs 0.0; 0.55 vs 0.01; 0.24 vs 0.0; 1.99 vs 0.67; all with a p-value of <0.05). While for the other markers, the concentration was significantly lower, including CO_2, NH_4, TVOC, NO_2, and acetone, in COVID-19 groups (respectively 607.3 vs 1175.1; 0.0 vs 1.05; 0.05 vs 146.6; 0.04 vs 1.55; 0.0 vs 0.23; all with a p-value of <0.05).

Table 2. Comparison between VOCs of exhaled breath between healthy subjects and COVID-19 subjects

Parameter		N	Mean	Std. Deviation	Median	p value
CO_2	Healthy	42	1278.5	610.9472	1175.14	0.000
	COVID-19	90	711.3599	348.57465	607.27	
	Total	132	891.8136	519.30829	891.21	
C_7H_8	Healthy	42	0.0167	0.06872	0	0.000
	COVID-19	90	0.8791	0.67732	0.93	
	Total	132	0.6047	0.68973	0.47	
CH_2O	Healthy	42	0.0453	0.10214	0.01	0.000
	COVID-19	90	1.7664	2.01069	0.55	
	Total	132	1.2188	1.84324	0.28	
NH_4	Healthy	42	0.9996	0.61911	1.05	0.001
	COVID-19	90	1.1749	2.00002	0	
	Total	132	1.1191	1.68651	0.52	
TVOC	Healthy	42	0.4158	0.59951	0.1466	0.000
	COVID-19	90	0.1313	0.20685	0.05	
	Total	132	0.2218	387.62139	0.080	
NO_2	Healthy	42	1.5615	0.76288	1.54	0.000
	COVID-19	90	0.0441	0.02336	0.04	
	Total	132	0.5269	0.82817	0.79	
CO	Healthy	42	0	0	0	0.000
	COVID-19	90	0.2298	0.07332	0.24	
	Total	132	0.1567	0.12326	0.12	
NH_3	Healthy	42	0.6637	0.32482	0.66	0.000
	COVID-19	90	2.08	1.3989	1.99	
	Total	132	1.6294	1.34202	1.32	
ACET	Healthy	42	0.2279	0.1536	0.23	0.001
	COVID-19	90	1.0751	1.99449	0	
	Total	132	0.8055	1.69319	0.11	

Definition of abbreviations: CO_2 = carbon dioxide; C_7H_8 = Toluene; CH_2O = Formaldehyde; NH_4 = Ammonium; TVOC = Total Volatile Organic Compounds; NO_2 = Nitrogen dioxide; CO = Carbon monoxide; NH_3 = Ammonia; ACET = Acetone

Further comparisons in COVID-19 groups was done to analyze the difference between VOCs's concentration based on the severity of the diseases. Only 6 markers show differences. CO (p-value 0.000), CO_2 (p-value 0.002), NH_4 (p-value 0.000), NH_3 (p-value 0.043), Acetone (p-value 0.002), and TVOC (p-value 0.000) were significantly different between subgroups based on disease severity. Correlation also shown between the severity of the disease and the concentration of those markers. The positive correlations found in NH_4, Acetone, CO, and NH_3

Table 3. Correlation of VOCs between subgroups based on disease severity in COVID-19 group

VOCs	Degree of severity	Median	Min	-	Max	p value
CO_2	Asymptomatic	603,4	462,67	-	1673,9	
	Mild	711,8	400	-	1876,5	
	Moderate	612,83	400	-	747,73	0,002*
	Severe	548,27	400	-	1443,4	
	Critically ill	431,03	400	-	1246,2	
TVOC	Asymptomatic	0,09	0,02	-	0,836	
	Mild	0,11	0	-	0,709	
	Moderate	0,02	0	-	0,15	0,000*
	Severe	0,02	0	-	0,194	
	Critically ill	0,01	0	-	0,071	
CO	Asymptomatic		0,14	±	0,06	
	Mild		0,20	±	0,07	
	Moderate		0,28	±	0,03	0,000*
	Severe		0,27	±	0,05	
	Critically ill		0,28	±	0,04	
NH_3	Asymptomatic		1178,89	±	634,10	
	Mild		971,46	±	644,57	
	Moderate		1398,91	±	289,86	0,043*
	Severe		1232,86	±	428,48	
	Critically ill		1250,63	±	392,80	
NH_4	Asymptomatic	0	0	-	10,19	
	Mild	0	0	-	3,43	
	Moderate	0,12	0	-	3,94	0,000*
	Severe	1,43	0	-	6,64	
	Critically ill	1,625	0	-	5,8	
ACET	Asymptomatic	0	0,00	-	8,17	
	Mild	0,00	0,00	-	1,64	
	Moderate	0,27	0,00	-	5,30	0,002*
	Severe	0,47	0.00	-	6,35	
	Critically ill	0,96	0,00	-	6,52	
NO_2	Asymptomatic		0,06	±	0,02	
	Mild		0,04	±	0,02	
	Moderate		0,04	±	0,03	0,275
	Severe		0,04	±	0,03	
	Critically ill		0,04	±	0,02	
C_7H_8	Asymptomatic	1,525	0,09	-	2,54	
	Mild	0,16	0,01	-	1,72	
	Moderate	1,105	0	-	1,82	0,243
	Severe	0,92	0	-	2,14	
	Critically ill	1,015	0	-	1,59	
CH_2O	Asymptomatic	4,4	0	-	7,16	
	Mild	0,02	0	-	5,14	
	Moderate	0,27	0	-	3,02	0,100
	Severe	1,49	0	-	4,64	
	Critically ill	2,245	0	-	4,71	

CO_2 and TVOC shows negative correlation. CO, NH_3, NH_4, and ACET show positive correlation. NO_2, C_7H_8, and CH_2O show no correlation.

with correlation coefficients were respectively 0.476, 0.358, 0.645, and 0.236. While the negative correlation is shown in CO_2 and TVOC with correlations coefficient -0.407 and -0.574.

DISCUSSION

Although the study related to the content of human breath has been conducted a long time ago, the first study recorded during the period 1777-1783 by Antoine Lavoisier, research in the medical field has not developed widely[9]. Using one of VOC as biomarker of disease is generally insufficient because of its complexity and heterogenous situation, including environmental exposures and the presence of chronic diseases. Detecting VOCs in exhaled breath has the potential for use as a diagnostic tool or a large-scale screening modality. Rather than detecting one VOC as a marker for one disease, using a set of VOCs and finding its pattern to create a "fingerprint" or "breath-print" is the preferred approach[10]. Our study found that a set of VOCs, consisting of C_7H_8, CH_2O, CO, NH_3, CO_2, NH_4, TVOC, NO_2, and acetone was able to differentiate between COVID-19 patients and healthy subjects.

Continuous monitoring of exhaled CO_2 is a method to ensure adequate ventilation during mechanical ventilation. The volume of CO_2 excreted by the cardiorespiratory system is a sensitive indicator of not only ventilation efficiency but also pulmonary perfusion and cardiac output[11]. Based on Enghoff-Bohr equation, a lower concentration of CO_2 can reflect the condition of increased dead space, ventilation – perfusion miss-match, and *Acute Respiratory Distress Syndrome* (ARDS). Those conditions

are usually occurred in COVID-19, and usually associated with its severity[12]. Low concentration of CO_2 in our research may be caused by those conditions. Using metabolic analyzers and volumetric capnography are preferable methods to measure CO_2 concentration and predict partial pressure of the mean expired CO_2[11].

Higher concentrations of NH_3 and lower NH_4 in COVID-19 patients, as well as a positive correlation with the degree of severity, may occur as a result of lower pH in the respiratory airway, acidification by gastric fluid, and influence of nitrite or nitrate metabolism by respiratory or gastrointestinal bacterial[13,14]. Previous studies of the airway pH of patients with ARDS failed to document acidification, although these patients had acidopnea[13]. Lower pH in the esophageal showed a higher expression of angiotensin-converting enzyme 2 (ACE2)[15]. Higher expression of ACE2 may responsible for the increased severity of COVID-19 and the risk of death by COVID-19[15,16].

The cytochrome P450 (CYP) expression and activity are greatly affected by an immune response and altered during COVID-19 infection[17]. The altered activity of CYP may lower the metabolism of C_7H_8 (toluene), causing higher excretion of toluene in exhaled breath[18]. Intracellular prooxidant / antioxidant imbalance leads to oxidative stress, resulting in lipid peroxidation[19]. Oxidative stress occurred during COVID-19 and increased levels of activated neutrophils increase the production of CH_2O[20,21]. Oxidative stress also induces heme oxygenase-1 (HO-1) activity in the airway, nasal epithelium, alveolar macrophages, endothelial cells, and other lung cell types, thus endogenously producing CO[22]. The concentration of CO also increases during infection, neutrophilic inflammation, and other critical conditions requiring mechanical ventilation[23]. Similar to CO, acetone may also increase as a result of infection, septic, and critically ill condition[24]. Treatment given during COVID-19 may also affect the concentration of VOCs. Antioxidants such as vitamin e and vitamin c, which are given in all COVID-19 subjects, are known to reduce the level of NO_2[25].

This study has some limitations. The first limitation is the use of a sensor-based analyzer can only calculate 1 type of VOC concentration per sensor. Thus, targeted VOCs are already pre-determined and other VOCs that may be pathognomonic for a disease, remain undetectable. The second limitation is many things can affect the results of research related to VOC. Variations of the sampling process and environmental influences such as exogenous VOCs, humidity, and temperature cannot be fully controlled. This may cause differences in results, between one study and another.

CONCLUSIONS

COVID-19 affects many aspects of the human body and it causes alteration of VOCs composition in exhaled breath. Our study concludes the detection of VOCs concentration can not only be used for diagnosis but also to assess the severity of the disease.

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