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Review Article

Plasmodium falciparum Breath Metabolomics (Breathomics) Analysis as a Non-Invasive Practical Method to Diagnose Malaria in Pediatrics

Ignatius Ivan[1], Maureen Miracle Stella[1], Stella Kallista[1], Kevin Tandarto[1], Fanny Budiman[1], Freggy Spicano Joprang[2]*

[1] School of Medicine and Health Sciences, Atma Jaya Catholic University, Jakarta, Indonesia
[2] Department of Parasitology, School of Medicine and Health Sciences, Atma Jaya Catholic University, Jakarta, Indonesia

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ABSTRACT

Children under 5 years of age are particularly vulnerable to malaria. Malaria has caused 445,000 deaths worldwide. Currently, rapid diagnostic tests (RDTs) are the fastest method to diagnose malaria. However, there are limitations that exist such as low sensitivity in detecting infections with low parasitemia. Practical, non-invasive and high ability tests to detect parasite are needed to find specific biomarkers for P. falciparum infection to determine the potential of P. falciparum 4 thioether in breathomics analysis by GC-MS as a practical non-invasive method in diagnosing malaria in pediatrics. Literature reviews from Google Scholar and ProQuest were published no later than the last 5 years. The concept of breathomics is that the breath's volatile organic compounds (VOCs) profile is altered when the health condition changes. Breath samples from individuals infected with P. falciparum malaria were taken by exhalation. Through GC-MS analysis, it was found that 4 thioether compounds (allyl methyl sulfide (AMS), 1-methylthio-propane, (Z) -1-methylthio-1-propene and (E) -1-methylthio-1-propene) underwent a significant change in concentration during the infection. Based on experiments conducted on mice and humans, the breathomics method is known to be able to detect parasitemia levels up to <100 parasites/µL, has a sensitivity level of about 71% to 91% and a specificity of about 75% to 94%. The discovery of 4 thioether compounds by GC-MS is a strong indication of malaria, because it has the potential for high sensitivity and specificity, and the detection power exceeds the ability of RDTs.

Keywords: *Breath metabolomics; malaria; Plasmodium falciparum; volatile organic compound*

ABSTRAK

Anak di bawah usia 5 tahun sangat rentan terkena malaria. Penyakit ini telah menyebabkan 445.000 kematian di seluruh dunia. Saat ini, rapid diagnostic tests (RDTs) merupakan metode yang paling cepat dalam mendiagnosis malaria. Namun, masih terdapat kelemahan seperti sensitivitas yang rendah dalam mendeteksi pasien dengan parasitemia rendah. Pemeriksaan praktis, non-invasif dengan daya deteksi parasit tinggi dibutuhkan untuk menemukan biomarker yang spesifik terhadap infeksi P. falciparum. Mengetahui potensi 4 thioether dari P. falciparum dalam analisis breathomics melalui GC-MS sebagai metode praktis non-invasif dalam mendiagnosis malaria pada pediatrik. Tinjauan pustaka dari Google Scholar dan ProQuest dengan kriteria rilis paling lama 5 tahun terakhir. Konsep breathomics adalah profil SOV dalam napas berubah ketika terjadi perubahan kondisi kesehatan. Sampel nafas dari individu yang terinfeksi malaria akibat P. falciparum diambil dengan menghembuskannya. Melalui analisis GC-MS, ditemukan bahwa 4 thioether (allyl methylsulfide, 1-methylthio-propane, (Z)-1-methylthio-1-propene, dan (E)-1-methylthio-1-propene) mengalami perubahan konsentrasi secara signifikan selama infeksi berlangsung. Berdasarkan percobaan yang dilakukan pada tikus dan manusia, metode breathomics diketahui mampu mendeteksi level parasitemia hingga <100 parasit/µL dan memiliki tingkat sensitivitas sekitar 71% hingga 91% dan spesifisitas sekitar 75% hingga 94%. Penemuan 4 senyawa thioether melalui GC-MS menjadi indikasi kuat terhadap penyakit malaria karena memiliki potensi tingkat sensitivitas dan spesifisitas yang tinggi, serta daya deteksi melebihi kemampuan RDTs.

* Corresponding Author:
freggy.spicano@atmajaya.ac.id

Kata kunci: *Breath metabolomics; malaria; Plasmodium falciparum; volatile organic compound*

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INTRODUCTION

Children under 5 years of age are one of the most vulnerable populations in malaria. This disease has caused 445,000 deaths worldwide.[1] Based on World Health Organization (WHO) data in 2017, the incidence of malaria in Southeast Asia is still quite high.[2] According to the latest World malaria report, released on 30 November 2020, there were 229 million cases of malaria in 2019 compared to 228 million cases in 2018. The estimated number of malaria deaths stood at 409 000 in 2019, compared with 411 000 deaths in 2018.[1] In addition, malaria is still a public health problem, because it can reduce the quality of life and increase the economic burden; furthermore, it has the potential to cause a plague. Malaria is caused by Plasmodium spp infection such as Plasmodium falciparum; the vector is the anopheline mosquitos such as Anopheles gambiae.[3]

Recently, malaria diagnostic tests were carried out by examining thick and thin blood smears, rapid diagnostic tests (RDTs), polymerase chain reaction (PCR) and serological tests (antibodies).[4] Currently, thick and thin blood smears are still the gold standard in diagnosing malaria. However, this examination requires a blood sample, which is an invasive process especially for pediatric patients.[5]

RDTs are currently the most effective method for diagnosing malaria on a large scale. However, RDTs have a low sensitivity in detecting asymptomatic patients; low levels of parasitemia may give inaccurate results due to cross-reactions to autoantibodies (such as the rheumatoid factor in the case of the HRP2 test).[4,5] In addition, this method has a limited shelf life, and somewhat qualitatively less sensitive than laboratory-based quantitative tests.[6]

Therefore, a non-invasive, fast and simple examination method is needed to increase the effectiveness and efficiency of implementing malaria diagnostic tests clinically. Technological advances have led to new techniques, namely the breath metabolomic test for biological sample research.[7,8] From 2010 to 2015, there were two types of metabolomic tests, invasive (using blood and tissue samples) and non-invasive (using saliva, urine, feces and respiration samples).[9] Although urine and fecal samples can be used as noninvasive biological samples, the sampling process might be uncomfortable, leading to instability of sample quality.[10] Breath sampling is another non-invasive method that does not require any discomfort.[11]

At present, non-invasive examinations through expiratory breathing (breath metabolomics/breathomics) are being developed.[9,10,11] In *P. falciparum* malaria patients, specific volatile organic compounds (VOCs) have been found in their inhalation-expiration, namely 4 thioether compounds (allyl methyl sulfide (AMS), 1-methylthio-propane, (Z)-1-methylthio-1-propene and (E)-1-methylthio-1-propene) which have never been established as another disease biomarker. The VOCs analysis method of exhalation is practical and beneficial due to its ability to detect low levels of parasitemia, which could not be done by RDTs.[9,10,11] In addition, this method is non-invasive and suitable for children and HIV/AIDS patients who are at high risk of developing opportunistic infections. It won't cause any discomfort and the results of the study can be obtained immediately.[12,13]

Through this review, the authors deliver the VOCs' potential of P. falciparum in breathomics analysis via GC-MS as a practical non-invasive method for detecting malaria, especially in pediatric patients.

The Role of Volatile Organic Compounds (VOCs) in *Plasmodium falciparum* as an attractant for *Anopheles*

Malaria parasites produce volatile signals recognized by mosquito vectors that attract mosquitoes to facilitate the transmission process. Vectors of malaria have special interest in infected individuals.[14] Several research projects have been conducted to study the chemical signals produced by the malaria parasite to attract the malaria mosquitoes to bite the host. This study aims to develop a non-invasive diagnostic tool to detect malaria in pediatric patients.

Studies of host and vector interactions show that several VOCs play an important role in attracting mosquitoes to infected humans.[14,15] The study used the headspace solid-phase micro-extraction/gas chromatography-mass spectrometry (HSPME GC-MS) analysis method on VOCs composition of extracellular vesicles and supernatants of ultracentrifugation (SNUs), performed on P. falciparum cultures at both high and low parasitemia levels.[16] The concentration of VOCs were detected by this method are 1,2,3-propanetriol and diacetate (diacetin). The supernatant analysis, however, gave off 56 VOCs, with pentane 2,2,4-trimethyl being present in all the SNUs of uninfected erythrocytes but absent from the parasite-infected ones.[16]

In both infected and uninfected individual red blood cells, 18 VOCs were obtained with elevated levels of 1,2,3-propanetriol, diacetate (diacetin) in infected extracellular vesicles. Diacetin is an insect-attracting compound found in plants.[15,16] Based on HSPME GC-MS analysis and supernatant, diacetin was found in the majority of infected erythrocytes and was found only once in uninfected erythrocytes. In supernatant analysis, 56 VOCs were found. Pentane 2,2,4-trimethyl was present in all SNUs of uninfected erythrocytes and was not found in infected erythrocytes.[17] Hexanal, a mosquito attractant compound, was the only VOC present in all samples from SNUs of infected erythrocytes. This shows that the component was formed when red blood cells were infected. This hexanal component is considered as an An. gambiae attractant for low malaria transmission.[18,19]

Although this VOC has been detected in Plasmodium vinckei culture in vitro, there are no reports of hexanal in *P. falciparum* infection.[20] These compounds may form during peroxidation of cell lipid membranes, and when red blood cells are under stress conditions such as when they were infected.[21]

Another study showed that mosquito attraction behavior can also be influenced by host odor. The study compared the chemical composition and attractiveness of *Anopheles coluzzii* to a *P. falciparum* infected individual's skin odor, because there were differences in the composition of the skin odor between infected and uninfected individuals.[22,23] These positive samples were collected within a mean time of two days after the parasites passed from the liver to the peripheral blood, with low parasite counts in the asexual phase and absence of gametocytes.[23] Several identified volatile compounds (2- and 3-methylbutanal, 3-hydroxy-2-butanone and 6-methyl-5-hepten-2-one) in this phase are known to influence mosquito behavior.[24] In addition, the skin microflora also plays a role in changing the composition of odor by increasing the production of 2- and 3-methylbutanal and 3-hydroxy-2-butanone emissions.[25]

Research showed that *An. gambiae* can respond to VOCs in the form of terpenes and their derivatives (10-carbon monoterpene such as pinene and limonene) at low concentrations produced by *P. falciparum*. Therefore, it can bite infected individuals to continue the malaria transmission process.[23,24] *An.gambiae* detects VOCs via signals that pass through a ligand voltage channel known as odorant receptors (AgORs). A VOC, which is a low

pressure isoprenoid and hydrocarbon compound, is produced by non-photosynthetic plastid organelles (apicoplast) via the methylerythritol phosphate (MEP) route during the intraerythrocytic period. In addition, plants also carry out the production process of terpenes by apicoplast. At low concentrations, terpenes can directly mediate the attractiveness of Anopheles spp.[24]

P. falciparum produces distinctive terpenes annotated as 15-carbon sesquiterpene (4,5,9,10-dehydroisolongifolene) and its close derivative (8,9-dehydro-9-formyl cycloisolongifolene). Additionally, each infected sample contained at least one 10-carbon monoterpene. This monoterpene annotation varies between samples, but is included in compounds containing the structural compounds limonene and pinanediol (alpha-pinene derivatives). [24]

To evaluate whether terpenes in samples infected with malaria parasites were produced de novo by the parasites, researchers used fosmidomycin, a phosphonic acid antibiotic that blocks the first specific enzyme of the MEP pathway, deoxyxylulose phosphate reductoisomerase. High terpene concentrations usually repel mosquitoes, while pinene and limonene at low concentrations can attract the attention of *An. gambiae*. This study shows that *P. falciparum* generates a repertoire of VOCs that serve as interspecies chemical signals that modulate the attractiveness of mosquitoes to hosts.[24,25]

Apart from terpenes, there are several other sodium orthovanadate SOV compounds in patients infected with malaria that have the potential to attract mosquitoes, namely 2- and 3-methylbutanal, 3-hydroxy-2-butanone, 6-methyl-5-hepten-2-one, carbon dioxide, isoprene, acetone, benzene, cyclohexanone, and 4 thioether, allyl methyl sulfide, 1-methylthio-propane, (Z)-1-methylthio-1-propene, (E)-1-methylthio -1-propene Methyl undecane, dimethyl decane, trimethyl hexane, nonanal, tridecane, α-pinene monoterpenes and 3-carene.[25]

Analysis of Volatile Organic Compounds (VOCs) in Vitro

In vitro analysis to detect VOCs based on the research of Wong et al used two methods, through solid phase micro-extraction (SPME) and purge and trap/thermal desorption (PTTD), which is connected to gas chromatography-mass spectrometry (GC-MS).[17,18] Trophozoite stage parasitemia with a level of > 5% were flowed into 1% hematocrit with 1% O_2 and 5% CO_2 environment via SPME. Furthermore, PTTD optimization is carried out so that the environment is more supportive of parasite life. This is done by circulating 5% O_2 and 5% CO_2, as well as maintaining the level of parasitemia at 1% hematocrit by 20%. This is compared to other methods that usually only maintain a 5% hematocrit parasitemia level of 5%.[17,18,19] In addition, the use of containers in the shorter PTTD method and wider base area also increases the mass of parasites and the resulting VOCs so that the parasite suspension can be increased from 18 mL (using a volumetric flask) to 50 mL.[17]

In the VOCs analysis through headspace analysis, there were 100 chemical compounds in the control group and the *P. falciparum* infected group. No specific biomarkers to identify *P. falciparum* were found in two groups. The VOCs obtained from the two methods showed no significant difference. There are no specific compounds that can be obtained from *P. falciparum* even though the analytical method used is different.[20]

Because of the limitations of this in vitro study, in vivo studies to detect VOCs from breathing are suggested to provide more specific SOV results that can be used as biomarkers of infectious disease.[17,21]

Profile of Volatile Organic Compounds (VOCs) in Malaria-Infected Mice

The evaluation topic of VOCs as an indicator of certain diseases using GC-MS has become popular among in vivo research.[25,26,27] SOV can be formed either due to parasite metabolism or host response to infection.[28]

This discovery later became the development of a diagnostic tool with the help of gas sensor arrays (GSA). GSA contains selective sensors formed from a collection of 11 quartz microbalance gas sensors (QMB) where the material consists of a solid layer of porphyrin.[28,29] The gas sample can then be flowed into this device via a miniature diaphragm pump.[27,28,29]

Based on the research, which uses GC-MS and GSA in mice infected with the *Plasmodium berghei*, parasite, VOCs were correlated with the infection.[27] Although parasite concentration did not have a strong correlation with the number of SOVs, this study focused more on the changes in VOC patterns in infected mice. The SOVs found from *P. berghei* infection were 2-nitro-1,4 benzenedicarboxamide, nonanal, 2,6-bis (1,1-dimethylethyl)-4-(1-oxopropyl) phenol and 2-methyl-2-propanamine.[28] Furthermore, in order to increase the ability of ASF in identifying Plasmodium infection in mice, the partial least squares discriminant analysis (PLSDA) algorithm was used, so that the sensitivity increased to 91% while the specificity increased by 75%.[28,29]

4 Thioether Compounds as Specific Expiratory Respiratory Specimens for Human Malaria Infection Biomarkers

Breath analysis offers an inexpensive and fast diagnostic test for several types of diseases. The results of the exhaled breath reflect the composition of the VOCs contained in blood. The concept of breathomics is that the VOCs composition profile in the breath changes when there is an infection or metabolic abnormality. The VOCs' composition of exhaled breath has the potential to assess not only the presence or absence of disease, but also the severity, progression and response to treatment. However, many aspects still exist at the methodological level that must be improved and optimized to achieve this goal.[30]

Exhalation results can be used as an alternative diagnostic sample compared to more invasive blood.[28,29,30] Based on a study, breath samples taken from individuals infected with *P. falciparum*, malaria can be used to detect the presence of typical VOCs associated with this infection.[30] The health worker will take a sample of the patient's breath by asking the patient to exhale a little and briefly (saying "ha"), then stop for a moment and continue the normal expiration on the cardboard tube between their lips until it's finished. By this method, the alveolar air is stored in the bag of the tool.[27,28] Then, using GC-MS, 9 compounds were found with varying concentrations during malaria infection, namely carbon dioxide, isoprene, acetone, benzene, cyclohexanone, allyl methyl sulfide, 1-methylthio-propane, (Z)-1-methylthio-1-propene and (E)-1-methylthio-1-propene.[30]

Among these compounds, 4 thioether compounds (allyl methyl sulfide, 1-methylthio-propane, (Z)-1-methylthio-1-propene and (E)-1-methylthio-1-propene) experienced a significant change in concentration during infection.[31] These four compounds have never been determined as biomarkers for other diseases, so they can potentially become specific biomarkers for *P. falciparum* malaria.[30,31] These four compounds are also reported to be low in healthy people.[32]

Berna *et al.* conducted a cohort experiment in 2 groups, where the difference was only in the administration of antimalarial drugs. The first group was given OZ439, which is a fast-acting synthetic drug, while the second group was given slower-acting piperaquine.[12] 4 thioether compounds might not be detected or barely detected when there was no infection. Four days later after the infection, the level of 4 thioether compounds increased and exceeded a maximum level of 6.5 hours after being given the drug.[12] The maximum level obtained after drug administration, indicates the presence of crushed erythrocytes (ruptured schizonts) so that merozoites spread out and infect the red blood cells, alongside parasite death, thus stimulating the maximum release of thioether.[13,14] Of the four thioether compounds for the cohort 1 group, (Z)-1-methylthio-1-propene reach the highest increase, 100 times at drug administered group than at initial period when there was no infection. Meanwhile, for cohort 2, 1-methylthio-propane reached the highest increase, about 90 times. After that, the level of VOCs in both cohort groups decreased

with clearance of parasitemia.[14,15]

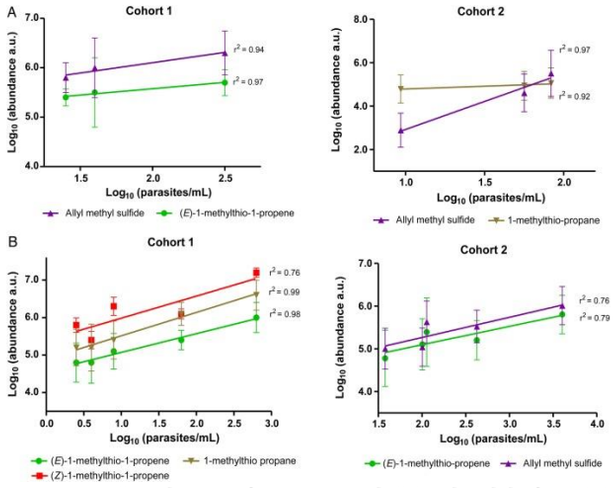

Figure 1. Correlation of parasite and SOV level before (A) using Allyl methyl sulfide and 1-methylthio-1-propene and after (B) using 1-methyltio-1- propene and 1-methylthio propane antimalarial treatment in 2 cohorts.[14] The thiother data were subjected to a phase shift of 24 hours, which revealed a direct correlation between parasitemia and volatile levels. In cohort 1, a fast-acting syhnthetic ozonide drug was used on day 7, and in cohort 2, a slower-acting piperaquine drug was administered on day 8. Points denote means and standard deviations.

As the result of the study above, it can be seen that the increase in VOC levels is directly proportional to the increase in blood parasitemia levels (Figure 1). In addition, the changes in VOCs of infected patients with low levels of parasitemia indicate that breath specimen analysis is a promising sensitive method in diagnosing malaria patients.[28,29,31] This method is able to detect parasitemia below the threshold level of rapid diagnostic tests against P. falciparum through PfHRP2 biomarkers (<100 parasites/μL).[31,32]

Another study conducted by Schaber et al., which analyzed breathomics using the breathprint method, was successful in diagnosing malaria infection in more than 80% of children with fever symptoms.[29] In addition, it shows 94% specificity and 71% sensitivity to Plasmodium falciparum. A higher sensitivity value is required to apply this diagnostic method widely. Nonetheless, these studies prove that breathprint analysis can be further refined to develop a safer and less invasive diagnostic tool. Such tests can be applied at a larger population level to monitor changes in malaria prevalence in endemic areas.[28,29]

Various Methods in Detecting 4 Thioether Compounds as a Breathomics Analysis Tool

Four thioether compounds that are *P. falciparum*-specific biomarkers can be detected using various techniques. Techniques include mass spectrometry (MS), e.g. gas chromatography-mass spectrometry (GC-MS), liquid chromatography-mass spectrometry (LC-MS) and proton transfer reaction mass spectrometry (PTR-MS); ion mobility spectrometry (IMS); differential mobility spectrometry (DMS); electronic nose device; colorimetric tests: infrared spectroscopy (IR spectroscopy), e.g. selected ion flow tube-mass spectrometry (SIFT-MS), fourier transform-infrared (FTIR) spectroscopy and ring-down cavity spectroscopy.[31,32]

Mass spectrometry interacts with ionizing molecules to produce charged molecules or molecular fragments; it further measures the mass to charge ratio, which can be used in conjunction with chromatographic separation techniques. LC-MS is an analytical method that combines the features of liquid chromatography and mass spectrometry to identify compounds. PTR-MS is a very sensitive technique for online monitoring of VOCs.[32] PTR-MS has high sensitivity (into the low pptv range) and a fast response time (in the 40-100 ms time regime). IMS is an analytical technique used to separate and identify ionized molecules in a gas phase, based on their mobility in a carrier buffer gas. This technique can be combined with mass spectrometry and/or chromatographic separation techniques.[33] DMS is distinguished by the difference between mobility in high and low electric fields because the value of ion mobility depends on the strength of the applied field. This method is easy to use, sensitive, fast and relatively selective. However, the use of mass spectrometry has several limitations, including the large size of the instrument and the difficulties to carry (non-portable). It is suggested that future device designs can be made more portable and easier to use by health workers clinically. In addition, processing sample data by mass spectrometry requires multiple data processing and analysis software,

as well as manual checking of raw data. This takes a long time, so it is recommended for researchers to develop simpler software to process the diagnosis results from breathomics.[34]

Currently, a portable version of mass spectrometry has been developed in a device called an electronic nose (E-nose) that detects the VOCs' composition of the breath sample.[35] This technique is also known as "electronic sensing" or "e-sensing". The E-nose includes three main parts: the sample delivery system, detection system and compute system. The detection system, which consists of a sensor set, is a 'reactive' part of the instrument. When in contact with VOCs, the sensor reacts and undergoes a change in electrical properties. On the E-nose, each sensor is sensitive to some volatile molecules, but each has a specific way and it depends on the sensor used.

This tool is a handful size so it's easy to carry everywhere. In addition, this tool can be used multiple times, then it is expected to have lower costs and more accurate and less invasive results compared to RDTs. This advantage makes this tool ideal to be used en masse, especially for screening.[36] Changes in the VOCs' composition of exhaled breath provide relevant information for diagnosing several diseases other than malaria, including cardiovascular disease, neurodegenerative diseases, oncogenic diseases, infectious diseases, diabetes mellitus and liver and kidney diseases.[37,38]

CONCLUSION

A practical, non-invasive method of examining malaria with a high level of sensitivity and specificity will be beneficial for a clinician to diagnose malaria, especially in pediatric patients. By using the VOCs' analysis method of exhaled gas (breathomics) from patients living in endemic areas with risk factors and clinical symptoms of malaria caused by Plasmodium falciparum, hopefully it can help to identify the *P. falciparum* parasite. The discovery of 4 thioether

CONFLICT OF INTEREST

There is no conflict of interest of this study.

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