ORIGINAL ARTICLE

Reproducible GC–MS Profiling of Urinary Metabolites as Biomarker Candidates for Dengue Infection: Seasonal Analysis Among Outpatients

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ABSTRACT

INTRODUCTION: Dengue diagnosis remains a clinical challenge as early symptoms might overlap with other febrile illnesses. Urine-based metabolic profiling offers a promising, non-invasive approach for detecting dengue infection biomarkers. In our earlier study among warded patients, gas chromatography mass spectrometry (GC-MS) identified nine urinary metabolites associated with dengue infection. This study assesses the consistency of these metabolites in non-warded outpatients. **MATERIALS AND METHODS:** A cross-sectional study was conducted at outpatient clinics in Kuantan involving 30 dengue-confirmed patients and 30 healthy volunteers. Midstream urine samples were collected prior to treatment, and dengue infection was confirmed through serological testing. The nine targeted metabolites were analysed using GC-MS method described in our previous study. **RESULTS:** Two metabolites (hexadecane and pentadecane) were consistently detected in dengue-positive patients but absent in controls. Hexadecane eluted at a retention time (RT) of 20.95 ± 2.23 min, with a spectral similarity index (SI) of 85.50 ± 5.00 % and a peak area of 1360566.25 ± 1066618.37 a.u. Pentadecane eluted at RT of 24.07 ± 3.35 min, with an SI of 86.00 ± 4.55 % and peak area of 853458.25 ± 523318.12 a.u. Hexadecane exhibited a stronger signal, approximately 1.6 times higher than pentadecane with 100% specificity and sensitivity of 8%. **CONCLUSION:** These findings confirm that the presence of urinary hexadecane and pentadecane remain consistent across different patient subgroups. These results provide preliminary evidence that urinary hexadecane and pentadecane are reproducibly detected in a subset of dengue patients and warrant further large-scale studies to confirm their diagnostic utility.

Keywords
Urinary, Biomarker, Dengue, Screening, Diagnostic

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INTRODUCTION

Dengue infection remains a significant public health concern in tropical and subtropical regions.[1] To date, no specific antiviral treatment exists for dengue fever; management remains primarily supportive, focusing on adequate hydration, rest, and relief of pain.[2] Its periodic outbreaks highlight the need for rapid, non-invasive diagnostic approaches. Laboratory diagnostic method for dengue virus infection is essential to support clinical diagnosis.[3] In the current clinical practice, several diagnostic approaches have been employed to confirm dengue infection including non-structural protein 1 (NS1) antigen detection, serological tests, molecular tests, and DENV subtype isolation by polymerase chain reaction (PCR).[4] Nonetheless, there is no single diagnostic method for dengue that fulfils the ideal criteria of sensitivity, specificity, speed, and affordability, and conventional tests often require venipuncture, trained personnel, and centralised facilities.[5] These limitations restrict their use in community or resource-limited settings. Moreover, only a limited number of studies have explored urinary

metabolites associated with this condition.[6–8] Urinary metabolic profiling with gas-chromatography mass spectrometry (GC-MS) has emerged as a promising alternative.[9] To our knowledge, this is among the first targeted reproducibility assessments to apply GC–MS analysis to previously identified urinary dengue-associated metabolites in outpatient dengue populations, with the goal of establishing a patient-friendly and non-invasive platform for identifying disease biomarker.

In our preliminary study, using an untargeted metabolomic approach via GC-MS among 10 hospitalised individuals diagnosed with dengue infection, we identified nine urinary metabolites associated with dengue infection namely, heptacosane, hexadecane, 2-bromooctane, tetradecane, pentadecane, 2,9-dimethyldecane, 2,4-bis (1,1-dimethylethyl) phenol, hexyl octyl ester sulphuric acid, and 2-benzoyl methyl ester benzoic acid.[10] Building on this, the present study evaluates the consistency and significance of these nine targeted urinary metabolites among non-warded patients and across multiple seasons. This approach aims to assess the robustness of these nine targeted urinary metabolites at different clinical settings and under natural temporal variations typical of tropical environments.

MATERIALS AND METHODS

Study Design and Participants

A cross-sectional study was conducted at outpatient clinics in Kuantan, from June 2022 to October 2023. Majority of the samples were collected between June to October each year, corresponding to non-monsoon season. Seasonal stratification was data-driven based on recorded sample collection dates rather than predetermined seasonal comparison. Inclusion criteria for the dengue-infected group comprised adults aged 18-60 years old with no known medical illness, newly diagnosed dengue infection confirmed by NS1 antigen and IgM/IgG serology, and ability to provide written informed consent. Exclusion criteria included individuals with chronic medical conditions (e.g., diabetes, hypertension, renal or hepatic disease), pregnant women, patients on long-term medication that could alter metabolic profiles, and patients with severe dengue (defined as dengue with any of the following clinical manifestations: severe plasma leakage causing shock or fluid accumulation with respiratory distress, severe bleeding, or severe organ impairment, including elevated transaminases ≥1,000 IU/L, impaired consciousness, or cardiac dysfunction). The control group consisted of adults aged 18–60 years with no known medical illness and capable of providing informed consent. Individuals with chronic diseases, pregnant or menstruating women were excluded.

Sample Collection

Midstream urine samples (15-30 mL) were collected in a sterile urine container and stored immediately at 4°C to preserve its integrity. Within 2 hours of collection, samples were centrifuged at 2000 rpm for 10 minutes to remove sediments. Post-centrifugation, 2 mL of the supernatant was carefully transferred into 1.5 mL microcentrifuge tubes (Eppendorf, Germany) and subsequently stored at -80°C, as described previously.[10] Urine of patients with suspected dengue infection were collected prior to treatment and dengue diagnosis was confirmed through serological test. The renal profile and liver function test were also performed to exclude underlying impairment and severe dengue.

The reproducibility of nine targeted urinary metabolites associated with dengue infection were analysed using GC-MS methods described in our initial study based on the retention time (RT) and similarity index (SI). Briefly, 1 µL of derivatized urine mixture was injected into a Shimadzu QP2010 Ultra GC-MS system (Kyoto, Japan) at an inlet temperature of 270°C using a 1:10 split ratio. Helium carrier gas was set at 1.2 mL/min. Separation was performed on an HP-5MS capillary column (30 m × 0.25 mm internal diameter, 0.25 µm film thickness) with the oven programme at 80°C for 2 minutes, ramped to 240°C at 5°C/min (held for 5 minutes), then to 300°C at 3°C/min (held for 5 minutes). The transfer line was maintained at 300°C. Electron ionisation was set at 70 eV with a mass range of 50–550 m/z. Metabolites were identified using the NIST 2017 library with similarity matches ≥80%. The levels of metabolites are quantified based on the peak area.[10]

Statistical Analysis

For each participant, GC-MS analysis was performed in three replicates, and the mean peak area of these replicates was used for all subsequent statistical comparisons. Data were analysed using IBM SPSS Statistics for Windows, Version 29.0 (IBM Corp., Armonk, NY, USA). Statistical significance was set at $p < 0.05$ with a 95% confidence interval. Categorical variables were expressed as frequencies and percentages, and comparisons were made using the Chi-square or Fisher's Exact test. Continuous variables with normal distribution were presented as mean ± standard deviation and analysed using the independent Student's t-test. Non-normally distributed data were expressed as median (interquartile range) and compared using the Mann–Whitney U test. Logistic regression was applied for multivariate analyses to adjust for covariates that might affect the production of urinary metabolites.

RESULTS

Sample Characteristics

30 dengue-confirmed patients and 30 healthy volunteers' urine samples were collected. There was no significant difference in the age, gender and ethnicity between dengue-infected subjects and healthy control group (p=0.523, 0.604 and 0.706 respectively) as shown in Table I.

Table I: Sociodemographic Characteristics of the Study Subjects (N=60)

Sociodemographic Characteristics		Overall (N = 60) n (%)	Dengue-infected subjects (n = 30) n (%)	Control group (n = 30) n (%)	p-value
Age group (years)	18-29	20 (33.3)	12 (40.0)	8 (26.7)	0.412*
	30-60	40 (66.7)	18 (60.0)	22 (73.3)	
Age (year)^		35.9 (12.6)	34.8 (11.6)	36.9 (13.6)	0.523**
Gender	Male	33 (55.0)	15 (50.0)	18 (60.0)	0.604*
	Female	27 (45.0)	15 (50.0)	12 (40.0)	
Ethnicity	Malay	52 (86.7)	27 (90.0)	25 (83.3)	0.706*
	Others	8 (13.3)	3 (10.0)	5 (16.7)	

^mean (SD), *Analysed using Chi-squared/Fisher's exact test, **Analysed using Independent Student's t-test

Both groups recorded a normothermic median temperature during the recruitment process. Dengue-infected subjects typically experienced fever for 4 days. The mean systolic blood pressure in dengue-infected subjects was significantly lower than controls (p=0.03). Nevertheless, they were within normotensive range. Other cardiovascular parameters such as pulse pressure, mean arterial pressure (MAP) and heart rate were similar in both groups as shown in Table II.

Table II: Clinical Parameters of the Study Subjects (N=60)

Clinical parameter	Overall (N= 60)	Dengue-infected subjects (n= 30)	Control group (n = 30)	p-value
Temperature (°C)#	36.7 (36.4 -37.1)	36.7 (36.5-37.2)	36.7 (36.4-37.0)	0.558*
Duration of fever (days)#		4 (3-6)	No fever	
Systolic blood pressure (mmHg)^	121 (17)	115 (14)	128 (17)	0.030**
Diastolic blood pressure (mmHg)^	77 (12)	75 (13)	79 (11)	0.254**
Pulse pressure (mmHg)^	45 (11)	48 (12)	41 (10)	0.201**
MAP (mmHg)^	92 (13)	95 (13)	89 (12)	0.076**
Heart rate (bpm)^	88 (16)	86 (16)	88 (15)	0.504**

#Median (IQR), ^mean (SD), IQR = interquartile range; MAP = mean arterial pressure; *Analysed using Mann-Whitney U/non-parametric test, **Analysed using Independent Student's t-test

Urinary Metabolites Detection

In the present study, five samples from the dengue-infected subjects and three from the control group were excluded in the GC-MS analyses due to compromised sample integrity, which resulted in error of the GC-MS analysis. From the remaining 52 samples, we found that two out of the nine targeted metabolites namely hexadecane and pentadecane (alkane group) were present in two samples (two to three replicates) of dengue-infected subjects. Hexadecane was characterised by a RT of 20.95 ± 2.23 min, a SI of 85.50 ± 5.00 %, and a mean peak area of 1360566.25 ± 1066618.37 a.u. Similarly, pentadecane exhibited a RT of 24.07 ± 3.35 min, a SI of 86.00 ± 4.55 %, and a mean peak area of 853458.25 ± 523318.12 a.u. The other targeted metabolites in this study were not detected in dengue-infected subjects. After evaluating the dataset comprising both positive and negative samples, it was observed that hexadecane and pentadecane demonstrated a sensitivity (true positive rate) of 8% and a specificity (true negative rate) of 100%.

Table III shows the statistical analysis of targeted metabolites identified in the study. The univariate analysis revealed presence of hexadecane and pentadecane in dengue-infected subjects (p=0.001 and p=0.024 respectively). Multivariate analysis further demonstrated the significant presence of hexadecane and pentadecane in dengue-infected subjects (p<0.01 and p<0.01) respectively.

Table III: Statistical Analysis of the Identified Targeted Metabolites (N=52)

Metabolite	Univariate analysis	Multivariate analysis	
	n = 156 (52 total subjects x 3 replicates)	n = 156 (52 total subjects x 3 replicates)	n = 75, (25 dengue-infected subjects x 3 replicates)
	p-value	p-value	p-value
Hexadecane	0.001*	<0.01**	<0.01**
Pentadecane	0.024*	<0.01**	<0.01**

*Analysed using Chi-squared/Fisher's exact test, **Analysed using Logistic Regression test
ϕAfter adjusting to presence of dengue infection, age, gender, ethnicity, day of fever, temperature, mean arterial pressure, heart rate, derangement of renal profile and liver function test

The two dengue patients with detectable urinary hexadecane and pentadecane were Malay females aged 22 and 43 years, recruited during the convalescent (recovery) phase, with normal renal function. One subject exhibited mildly elevated alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels, while the second subject exhibited liver parameters within normal ranges.

DISCUSSION

Subjects Socio-Demographics

Our study found no significant differences in age, gender, or ethnicity between dengue-infected subjects and the healthy control group. Approximately 60% of dengue-infected participants were aged 30–60 years, consistent with previous local reports showing higher susceptibility in this age group, likely due to increased outdoor exposure related to occupational and recreational activities.[11]

Our dengue-infected subjects were represented by a relatively equal number of males and females. In contrast, dengue infections are reported to be more prevalent among males, attributed to differences in gender-related behaviours and activities that increase exposure to the virus.[12,13] However, our observation may not accurately reflect the actual population, as the number of participants from both genders at the time of recruitment was roughly equal. Furthermore, our data showed that 90% of dengue-infected participants were Malays, reflecting the predominantly Malay demographic composition of the study area. It is important to note, however, that dengue infection does not demonstrate ethnic selectivity, as it affects Malay, Chinese, and Indian populations equally.[14]

Clinical Parameters

The present study revealed no significant difference in both groups of study subjects' body temperature, heart rates, and renal profile. The mean systolic blood pressure in dengue-infected subjects was within normal range despite significantly lower than controls.[15] Our study findings indicated that the dengue infected subjects were in the later stage of dengue infection as evidenced by positive IgM and IgG detected at their first presentation at healthcare facility, and were experiencing a non-severe dengue infection, which did not significantly impact the renal profile when compared to the controls. Furthermore, all exhibited no fever and maintained normal clinical parameters. The derangement of liver function test (raised ALT and AST) of one dengue-infected subjects could be explained by findings of other studies that hepatocellular injury is prevalent in more than half of the dengue-infected individuals.[16] This further highlights the necessity of liver enzymes serial monitoring throughout the course of the disease.

Detection of Targeted Urinary Metabolites

Both urinary metabolites *viz* pentadecane and hexadecane detected in serologically confirmed dengue-infected patients, belong to the alkane group. Dengue virus lacks metabolic enzymes capable of catalysing alkane oxidation.[17] Other studies found that dengue infection induces activation of immune and endothelial cells, leading to excessive generation of reactive oxygen and nitrogen species (ROS/RNS). These oxidants trigger lipid peroxidation of cell membranes, reflected by elevated markers such as malondialdehyde (MDA), 4-hydroxynonenal (4-HNE), and protein carbonyls, and result in the release of volatile hydrocarbons including alkanes, aldehydes, and ketones.[18–20]

Notably, positive detections of both urinary metabolites occurred in patients sampled during the convalescent phase. One plausible explanation is that urinary excretion of volatile hydrocarbons related to oxidative membrane injury may be detectable in time-dependent manner after the peak febrile period, when viraemia and acute systemic symptoms are subsiding. In addition, outpatient presentation in later illness may have limited our ability to

capture acute-phase metabolic markers. In contrast, humans do not utilise alkanes as energy substrates. Instead, cytochrome P450-mediated oxidation, can act on hydrocarbon substrates and occupational alkanes, producing alcohol, aldehyde, and acid intermediates that are subsequently excreted.[21] Together, these oxidative and detoxification mechanisms provide a plausible explanation for the urinary detection of hexadecane and pentadecane in dengue patients, reflecting host-derived oxidative membrane injury.

In comparison, bacterial species such as *Pseudomonas aeruginosa, Mycobacterium tuberculosis* and *Acinetobacter spp.* can produce similar alkanes through alkane monooxygenase (AlkB)-mediated oxidation.[22] In this study, however, the absence of bacterial growth and the temporal association with viremia suggest that these urinary alkanes are endogenously generated by the host. Collectively, our findings indicate that urinary pentadecane and hexadecane are host-derived oxidation products reflecting intense oxidative stress and membrane lipid breakdown triggered by dengue virus infection. These metabolites may therefore serve as non-invasive biomarkers of oxidative injury, potentially correlating with disease severity and tissue damage. Future metabolomic investigations should correlate urinary alkane profiles with clinical outcomes, hepatic function, and oxidative biomarkers to enhance their diagnostic and prognostic relevance.

CONCLUSION

This study suggests that urinary hexadecane and pentadecane levels remain consistent in a subset of dengue outpatients and are in-line with an intrinsic oxidative host response, reflecting a plausible mechanistic link between dengue-associated lipid peroxidation and urinary volatile alkane excretion. Importantly, these findings demonstrate the feasibility of targeted urinary metabolite detection using GC–MS in an outpatient setting. However, given the low detection rate observed, these metabolites should be regarded as exploratory biomarker candidates requiring further phase-stratified and larger-scale validation before any diagnostic application.

LIMITATIONS AND FUTURE DIRECTIONS

Nonetheless, several limitations should be acknowledged. The low sensitivity of urinary hexadecane and pentadecane detection despite high specificity suggests that most dengue-confirmed outpatients did not exhibit detectable levels of these metabolites under current sampling and analytical conditions, limiting their immediate utility as standalone diagnostic biomarkers. In addition, both detections occurred during the convalescent phase indicate that metabolite detectability may differ across stages of illness and that the optimal diagnostic window has yet to be established. The sample size was modest, and recruitment was limited to a single geographic region further limit generalisability. Quantitative validation using targeted metabolomics and controlled comparisons with other viral or bacterial infections are needed to confirm the specificity of hexadecane and pentadecane as dengue-related oxidative markers.

Future studies should integrate phase-stratified (acute, critical, and recovery phases), clinical severity grading and larger multicentre cohorts to clarify specificity, temporal dynamics and prognostic value of these metabolites in dengue infection. By addressing these areas, we aim to strengthen the evidence base for non-invasive urinary metabolite profiling as a feasible approach for dengue biomarker development.

INSTITUTIONAL REVIEW BOARD (ETHICS COMMITTEE)

This study was approved by the institutional, IIUM Research Ethics Committee (IREC) [IREC 2019-187]. Written informed consent was obtained from all participants.

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