BIOMARKER IDENTIFICATION FROM URINE OF DENGUE PATIENTS THROUGH GCMS FOR INITIAL DEVELOPMENT OF NON-INVASIVE DIAGNOSTIC KIT

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ABSTRACT: Dengue infection detection methods, namely dengue serology test and Real-Time PCR, are only available in clinical laboratories or healthcare facilities. This is time-consuming and inconvenient for patients. Thus, a non-invasive on-site urinary diagnostic kit for dengue infection that requires no trained personnel for blood extraction would be advantageous and warranted, albeit in developed or underdeveloped nations with limited resources. To develop the on-site diagnostic kit, an identification of biomarkers related to dengue infection is needed. This preliminary study aims to identify potential metabolite biomarkers from the urine of dengue patients using GCMS analysis. Urine samples of patients with serologically confirmed dengue infection were analyzed and compared with healthy volunteers. The study shows that the GCMS approach can identify differences in the urine of dengue patients from healthy volunteers. Heptacosane, Hexadecane, 2,4-bis(1,1-dimethylethyl) phenol, 2-bromooctane, tetradecane, hexyl octyl ester sulfurous acid, 2-benzoyl methyl ester benzoic acid, 2,9-dimethyl decane, and pentadecane were identified from the urine of dengue patients. The identified secretion of alkane may be a suitable candidate for colorimetric assay for the development of a user-friendly, home-screening rapid test kit for the detection of dengue infection.

ABSTRAK: Kaedah pengesanan jangkitan denggi seperti ujian serologi denggi dan PCR masa nyata, merupakan kaedah biasa yang digunakan di makmal klinikal atau pusat kesihatan. Ianya memakan masa dan menyukarkan pesakit. Oleh itu, kit diagnostik air kencing yang tidak invasif yang tidak memerlukan kakitangan terlatih bagi mengekstrak darah adalah sangat berguna dan diperlukan bagi mengesan jangkitan denggi, sama ada di negara maju atau tidak membangun dengan sumber terhad. Bagi membangunkan kit diagnostik ini, pengesanan penanda bio berkaitan jangkitan denggi diperlukan. Kajian awal ini bertujuan bagi mengenal pasti penanda bio metabolit yang berkaitan pada air kencing pesakit denggi dengan menggunakan analisis GC/MS. Sampel air kencing pesakit yang disahkan secara serologi telah dikaji dan dibandingkan dengan sukarelawan yang sihat. Kajian menunjukkan teknik GCMS dapat mengenal pasti perbezaan antara air kencing pesakit denggi dan sukarelawan yang sihat. Heptakosane, Hekzadekane, fenol 2,4-bis(1,1-dimetiletil), 2-bromookten, tetradekana, asid sulfurik ester heksil oktil, asid benzoik metil ester 2-benzoik, 2,9-dimetil

dekana, dan pentadekana telah dikenal pasti terkumpul dalam air kencing pesakit denggi. Rembesan alkana merupakan penanda bio yang sesuai bagi ujian kolorimetrik dalam membangunkan kit ujian pantas diagnostik yang mesra pengguna bagi mengesan denggi di rumah.

1. INTRODUCTION

Dengue is the most prevalent arboviral infection globally, affecting 400 million people worldwide every year [1]. It is caused by the dengue viruses (DENVs) that are transmitted by its' principal vector, the *Aedes aegypti* mosquitoes [2]. It is endemic in tropical and subtropical areas where the *Aedes sp.* is highly infested. Of note, Asian countries score the highest dengue disease burden internationally [2-4]. It was reported that over the past 50 years, the incidence of dengue has increased by 300% globally [3,4]. This trend is mainly attributed to global warming, population growth, urbanization, globalization, and enhanced human mobility, as well as inadequate mosquito control measures [1-3]. In Malaysia, dengue incidence was reported to be between 69.9 and 93.4 per 1000 people [4].

1.1. Pathophysiology

Dengue infection has a heterogeneous manifestation ranging from asymptomatic to mild, self-limiting febrile illness to severe and life-threatening systemic disease, depending on the viral-host interplay [2,3]. The three stages of dengue include the incubation phase, the febrile phase, and the critical phase [2]. DENV is a member of the Flaviviridae family with four well-described serotypes of dengue viruses: DENV1-4 [1-3]. DENV is a single-stranded RNA virus that encodes for the capsid, pre-membrane, envelope, and seven non-structural proteins, all of which contribute to its virulence and were the target of vaccine development [1, 5]. Infection by one serotype provides long-term immunity towards the specific serotype and some degree of cross-protection against the other serotypes [1]. Surprisingly, this cross-protection was reported to increase the risk of severe dengue in secondary infection by other serotypes [1,3,6]. This antibody-dependent enhancement phenomenon has proven to be detrimental as it augments viral entry into the immune cells, escapes the immune responses, and dysregulates the pro-and anti-inflammatory balance[1-2]. Eventually, this leads to dengue shock syndrome, which could be fatal.

1.2. Dengue Infection Method of Detection

At present, the dengue serology test is the gold standard for confirming the diagnosis by detecting anti-DENV IgM and IgG [7]. Other methods, such as virus isolation and molecular approach using reverse transcriptase-polymerase chain reaction (RT-PCR), have also been used in detecting the presence of the dengue virus in a blood sample [8]. However, all these methods are laboratory-dependent, require blood extraction, and are time-consuming, which may cause a delay in diagnosis. Moreover, the requirement for blood extraction procedures may not be the preference of some patients. Hence, these methods are more practical for research purposes and as diagnostic tools in healthcare facilities. Moving forward, several rapid diagnostic tests have been established to help physicians make quick diagnoses [9]. Although the rapid diagnostic test kit may not be as accurate as the standard test, the kit remains useful for initial patient screening, particularly in limited-resource hospital settings and remote areas [10]. Nonetheless, blood extraction is still required for these rapid diagnostic test kits.

Alternative samples, such as urine and saliva, have been tested [11]. In one study, dengue RNA was detected in serum, urine, and saliva samples using real-time RT-PCR for DENV

detection and quantification [11]. Another study analyzed a larger sample size of urine samples and concluded that RT-PCR and NS1 tests demonstrated an overall sensitivity and specificity of 41.6%/ 14.5% in urine, as compared to 85.4%/ 63.4% in plasma [12]. A year later, the same authors evaluated the use of urine samples in several commercialized rapid diagnostic kits and demonstrated promising data [13]. However, the authors recommended that the sensitivity of the kit be further improved for urine samples before utilizing it in clinical settings. Therefore, the use of urine as the sample for the initial screening method before final detection is promising.

1.3. Dengue Infection Diagnostic Challenges

To date, there is no specific treatment for dengue infection; management is merely supportive [7]. Debilitating complications of dengue infection stresses the importance of early detection and management to reduce morbidity and mortality rates. Diagnostic delay in dengue infection can be related to physician and patient factors. Physicians or travelers from non-endemic regions may have low suspicion of dengue infection, contributing to the delay. Patients' delay in seeking treatment may also contribute to the development of disease complications. Among the factors associated with delayed care-seeking is poverty, which may affect one's behavior despite the available resources [14]. Interestingly, one study in Thailand found that being female is significantly associated with morbidity among dengue patients [15]. These associated factors may imply that the inconvenience of performing the diagnostic test, which is only available in a bigger healthcare facility, causes patients to put off seeking treatment until more serious symptoms develop.

Presently, gold-standard diagnostic tests for dengue involve invasive blood extraction from suspected patients [7–9]. Blood-collecting procedures could be difficult in some dengue patients, especially among those presenting with hemorrhagic shock, children, and newborns. Furthermore, patients with typanophobia or extreme fear of medical procedures involving needles could delay seeking treatment [15]. Hence, an alternative for a non-invasive method using other than blood samples, which require minimal skills to perform, is very much needed. A diagnostic kit using salivary or urinary samples helps to remove the hindrance associated with typanophobia apart from providing options.

Therefore, this study aims to explore the potential of developing a non-invasive urinary diagnostic kit for dengue infection that would be advantageous and warranted, albeit in more developed or underdeveloped nations with limited resources. Together with continuing education to raise awareness of its early symptoms, a sensitive, feasible, and affordable diagnostic tool will enable early initiation of supportive treatment and may reduce morbidity and mortality among dengue patients

2. MATERIALS AND METHOD

All chemicals and reagents purchased are analytical grades.

2.1. Sample collection

Ten urine samples were obtained from patients with serologically confirmed dengue infection who were admitted to IIUM Sultan Ahmad Shah Medical Centre, Kuantan. Patients with a history of chronic diseases or pregnant women were excluded. Forty milliliters of urine samples were collected at 8 a.m. in all consented patients aged 18 - 60 years. An additional 3 urine samples from healthy volunteers were also obtained. The urine was centrifuged for 10 minutes at 3000 revolutions per minute within 2 hours of collection. The supernatant was aliquoted and stored in a -80°C freezer to prevent metabolite changes before analysis. Ethical

approval was obtained from the International Islamic University Malaysia Research Ethics Committee (IREC 2019-187).

The urine samples were registered as C1, C2, C3, D1, D2, D3, D4, D5, D6, D7, D8, D9, and D10 then subjected to freeze-drying activity including main-drying under 0.011 mbar at -60°C for 48 h and final-drying under 0.011 mbar at -76°C for another 48 h.

2.2. Sample preparation

An amount of 0.01 g freeze-dried urine samples was derivatized with 0.5 mL of N,O-bis-trimethylsilyl (BSTFA)-trifluoroacetamide-trimethylchlo-rosilane (TMCS) mixture at 99:1 ratio (Macherey–Nagel, Germany) and 0.5 mL hexane to give a 0.01 g/mL urine mixture. The mixture was then heated at 60°C for 30 min before injection of 1 µL urine mixture into the Pegasus High Throughput Gas Chromatography Time-of-Flight Mass Spectrometer (GC-TOF/MS) [16].

2.3. GC/MS Analysis

A volume of 1 µL urine mixture was injected into the 225°C GC-TOF/MS (Leco, Netherlands) inlet at a 1:10 splitless mode ratio. The urine mixture was evaporated and carried at 1.2 mL/min rate by He gas to the oven at temperature programming: 80°C for 2 min; raised to 240°C at 5°C/min for 5 min, and raised to 300°C at a rate of 3 °C/min for 5 min. The evaporated metabolites of the urine mixture were separated by an HP-5 MS capillary column (30 m × 0.25 mm internal diameter and 0.25 µm film thickness), eluted to 300°C transfer line of the mass spectrometer (MS), and detected by the MS set at 70 eV electron energy and a mass range of 50–550 m/z. The detected metabolites were compared to the National Institute of Standard (NIST) Library 2017, where compounds with similarities of more than 80% were chosen for multivariate data analysis [17]. At least five replicates of the GCMS analysis were carried out for each sample. The composition of each urine was reported in the form of percentage composition.

2.4. Dataset pre-processing

The percentage composition of each urine sample was imported to the dataset table in XLSTAT 2019 software (Addinsoft, Paris, France). About 65 urines x 46 metabolites in the dataset were pre-processed to facilitate the process of differentiation among the urines. This pre-processing step is applied to reduce the dataset's variation of metabolites. Dataset pre-processing tests consisted of dataset transformation using XLSTAT-Pro (2019) statistical software.

To ensure the dataset follows normal distribution before the PCA, the dataset normality was tested using Lilliefors at α of 0.05. The dataset was transformed using standardize (n-1), standardize (n), center, standard deviation−1 (n-1), standard deviation−1 (n), rescale from 0 to 1, rescale from 0 to 100, and Pareto methods. The transformation of each metabolite was employed to ensure the transformed dataset remained closer to the original dataset. The normal distribution of the transformed dataset was evaluated by the normality test of Lilliefors at α of 0.05 [18].

2.5. Principal component analysis

The PCA was applied to investigate the metabolite contribution to the urines and identify the variance of intercorrelated metabolites at α of 0.05. These objectives were achieved by transforming the dataset into principal components (PCs), which are new sets of independent variables that are displayed in the form of correlation biplot and metabolite plot.

In this study, cumulative variability (CV) of two-dimensional PCs entailing PC1 and PC2 was computed for the metabolite exploratory. The number of PCs selected was based on the highest cumulative variability. The metabolites with $FL \geq |0.750|$, $|0.500| < FL < |0.749|$ and $FL \leq |0.499|$ were considered metabolites with strong, moderate, and weak factor loading, respectively. To determine the contribution of metabolites to the urines, the metabolite and correlation biplots were plotted, and the metabolites that contributed to each urine were examined and further explained. The metabolites with weak FL were removed from the metabolite dataset. Using 21 metabolites with strong and moderate FLs, a new PCA was executed to produce a new metabolite plot and correlation biplot. The FL and correlations of the metabolites were assessed, and the metabolites' apportionment to the urine was examined [18].

2.6. Discriminant analysis

In this study, discriminant analysis (DA) was employed to develop a discriminating model (DM) for the urines. Only the urines of dengue-infected patients were present at $F1 > 0$, and control urine was present at $F1 < 0$, which brought the dataset to 26 urines x 21 metabolites as both training and cross-validation datasets. An assessment of the classification of urine in the training dataset was performed. The correct classification of the dengue-infected patients and controls was assessed, and their dissimilarity was explored. Classification at $< 100\%$ for the training dataset indicated potential urines and metabolites that may render incorrect discrimination between dengue-infected and controls; hence, these urines and metabolites were removed, and discriminant analysis was repeated. After obtaining 100% correct classification in training datasets, the developed DM was cross-validated using the same dataset to validate the high predictive ability [19]. Then, the significant metabolites that contributed to the DM were identified via a unidimensional test of means equality of the dengue-infected patients and controls.

3. RESULTS AND DISCUSSION

3.1. Chemical composition of urine from dengue-infected patients

The chemical composition of urine from dengue-infected patients is listed in Table 1.

3.2 Metabolite correlations

The Pearson correlation test was chosen to select suitable correlation types to determine correlations between two metabolites. The correlation explores the dataset in investigating the strength (weak, moderate, or strong) and direction (positive and negative) of the linear relationship between two metabolites. Table 2 shows the correlation matrix (CM) of the metabolites in the urine of dengue-infected patients. The CM measures the strength and direction of a linear relationship between two metabolites. The CM indicated that positive and negative correlations among the metabolites at $|0.700| < R < |1.000|$ for strong CM, $|0.300| < R < |0.700|$ for moderate CM, and $3|0.000| < R < |0.300|$ for weak CM as in generic guideline.

Table 1. Chemical composition of urine from dengue-infected patients

Metabolite	Retention time, s	Composition of metabolites in the urine of dengue-infected patient[1], %												
		C1	C2	C3	D1	D2	D3	D4	D5	D6	D7	D8	D9	D10
Allyl octyl ester oxalic acid	494.603	nd	nd	nd	0.16	nd	nd	nd	nd	nd	1.83	nd	nd	0.70
2,4,4-trimethyl hexane	495.002	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
3,3-dimethyl hexane	507.319	nd	nd	1.31	nd	nd	nd	1.38	5.72	nd	nd	nd	0.48	1.12
2,3,3-trimethyl pentane	530.824	nd	nd	1.25	nd	nd	0.20	nd	0.63	nd	nd	nd	0.39	nd
2,9-dimethyl decane	542.?42	nd	nd	nd	nd	0.85	nd	0.37	1.22	nd	nd	nd	0.26	0.41
Dodecamethyl cyclohexasiloxane	607.052	nd	5.12	2.86	9.55	1.95	8.63	0.64	12.53	2.66	0.44	nd	2.23	nd
2-methyl dodecane	792.872	nd	nd	1.16	nd	nd	nd	nd	nd	nd	nd	nd	0.51	0.15
Tetradecane	792.904	nd	nd	nd	0.64	nd	nd	1.05	nd	5.21	0.88	nd	2.37	1.89
Hexyl pentyl ester sulfurous acid	793.138	nd	nd	1.68	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
1-iodo tetradecane	801.936	nd	nd	nd	nd	nd	nd	nd	1.56	3.60	0.16	nd	1.01	0.28
2,3,5,8-tetramethyl decane	832.878	nd	nd	7.97	nd	4.17	nd	4.66	nd	3.93	9.59	nd	11.15	19.23
2,2,5-trimethyl 3,4-hexanedione	840.846	nd	nd	nd	nd	4.62	nd	nd	0.31	nd	nd	nd	nd	0.20
Hexadecane	840.946	26.00	2.43	0.98	5.08	1.69	6.96	10.12	2.09	6.30	23.06	nd	14.53	11.94
Nonadecane	840.960	6.37	41.26	0.67	4.98	25.92	14.71	13.38	21./1	3.64	2.70	nd	1.71	0.50
4-methyl-1-undecene	840.979	3.13	nd	4.49	22.11	0.34	nd	2.30	3.58	5.82	1.48	nd	3.73	3.69
2,6-dimethyl heptadecane	841.543	2nd	nd	4.63	nd	4.08	nd	1.93	nd	17.28	nd	nd	2.25	0.55
3-isopropoxy-1,1,1,7,7,7-hexamethyl-3,5,5-tris(trimethylsiloxy)tetrasiloxane	855.985	nd	nd	2.26	9.25	2.42	nd	0.84	1.38	nd	nd	nd	nd	nd
2,4-bis(1,1-dimethylethyl) phenol	864.617	3.89	6.48	30.04	7.06	7.04	3.61	4.62	nd	1.74	2.04	nd	1.22	9.33
Allyl nonyl ester oxalic acid	903.328	0.35	nd	nd	0.22	0.35	4.22	nd	nd	2.10	0.27	nd	3.57	3.44
2,6,11-trimethyl dodecane	903.395	2.41	nd	nd	nd	11.56	nd	nd	nd	1.70	nd	nd	11.06	0.16
1-iodo-2-methylnonane	903.410	nd	8.15	0.73	5.91	2.93	7.76	2.81	0.44	1.95	1.67	nd	1.90	0.58
Tridecane	903.461	3.72	nd	1.57	nd	nd	nd	nd	nd	nd	0.02	nd	1.18	0.12
2-bromooctane	983.540	nd	nd	0.92	nd	nd	nd	0.49	nd	0.28	nd	nd	0.83	0.66
(1,1-dimethylethyl)thio acetic acid	1030.880	nd	nd	3.25	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
2,4-dimethyl undecane	1031.210	nd	1.40	nd	nd	nd	nd	nd	nd	2.11	nd	nd	0.70	0.64
Octyl propyl ester oxalic acid	1098.540	nd	0.64	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	1.52
Hexyl octyl ester sulfurous acid	1128.160	nd	nd	nd	1.67	nd	nd	1.56	0.69	1.31	nd	nd	0.45	0.08
Eicosane	1128.230	5.33	nd	2.52	3.32	6.23	7.52	3.24	4.27	4.77	15.36	nd	7.45	3.91
4,7-dimethyl undecane	1128.280	nd	nd	1.97	5.63	0.38	nd	1.55	4.02	nd	nd	nd	0.35	0.15
Undecane	1128.320	5.04	nd	nd	nd	nd	nd	nd	nd	nd	0.51	nd	0.77	0.23
Dotriacontane	1128.360	nd	nd	10.43	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
1-iodo-2-methylundecane	1128.390	4.78	10.06	9.78	11.68	5.01	35.87	1.18	11.51	1.80	3.01	nd	6.37	12.06
2,6,10,14-tetramethyl heptadecane	1138.920	nd	nd	nd	0.27	1.10	nd	0.52	nd	1.32	0.44	nd	0.34	0.46
Pentadecane	1183.540	3.08	5.82	6.12	4.31	7.70	8.88	16.65	7.79	3.48	10.80	nd	7.53	11.02

Metabolite	Retention time, s	Composition of metabolites in the urine of dengue-infected patient[1], %											
		C1	C2	C3	D1	D2	D3	D4	D5	D6	D7	D8	D9	D10
2,4,6-trimethyl octane	1183.700	5.33	1.72	nd	0.82	1.59	nd	3.56	nd	nd	3.08	nd	1.22	2.81
Heptadecane	1183.870	nd	6.25	nd	nd	nd	nd	2.84	9.22	5.76	1.94	nd	2.71	0.55
N,N,O-triacetylhydroxylamine	1195.060	nd	nd	nd	0.35	nd	nd	nd	nd	nd	nd	nd	nd	nd
Hexacosane	1388.580	nd	2.08	nd	nd	nd	nd	1.66	nd	3.92	nd	nd	1.26	nd
2-ethylhexyl isohexyl ester sulfurous acid	1388.610	2.70	nd	nd	nd	nd	nd	3.51	0.48	4.16	1.61	nd	0.15	0.45
1-iodoundecane	1388.630	nd	nd	0.55	0.10	0.85	1.85	nd	nd	0.17	nd	nd	0.73	0.13
2,2,3,3,5,6,6-heptamethyl heptane	1388.650	nd	1.78	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
2-ethylhexyl hexyl ester sulfurous acid	1388.680	nd	nd	2.42	nd	0.84	nd	nd	nd	2.36	nd	nd	nd	nd
Hexatriacontane	1388.750	nd	3.54	nd	nd	nd	nd	0.21	nd	nd	nd	nd	nd	nd
2-methyl heptadecane	1388.940	1.34	nd	0.45	5.89	4.96	nd	10.46	6.15	nd	nd	nd	5.70	6.15
2-benzoyl methyl ester benzoic acid	1392.830	nd	nd	nd	1.02	2.75	nd	2.66	3.35	3.76	8.33	nd	1.25	3.14
Heptacosane	1437.420	6.53	3.26	nd	nd	0.65	nd	5.59	1.98	8.23	10.77	nd	2.66	1.74

Note: [1]nd = not detected

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Table 2. Correlation matrix of metabolites in the urine of dengue-infected patients

Metabolites[1]	Correlation matrix, CM[2,3]																					
	M3	M4	M5	M6	M8	M9	M11	M13	M14	M15	M17	M22	M28	M31	M32	M35	M39	M40	M41	M43	M45	M46
M3	1.000	0.540	0.412	0.242	0.637	0.582	0.402	**0.707**	0.603	0.302	**0.769**	0.344	0.236	0.284	**0.785**	0.511	0.459	**0.753**	0.220	**0.775**	0.542	0.222
M4	0.540	1.000	**0.723**	-0.049	0.627	0.199	0.220	0.410	0.190	0.304	0.575	0.255	-0.042	0.296	**0.783**	0.700	0.552	**0.749**	0.664	0.570	**0.906**	0.374
M5	0.412	**0.723**	1.000	0.070	0.585	0.192	0.286	0.495	0.104	0.570	0.533	0.473	-0.006	0.137	**0.748**	0.584	0.360	**0.871**	0.149	0.556	0.218
M6	0.242	-0.049	0.070	1.000	0.003	0.109	-0.061	0.140	0.013	-0.040	0.001	**0.737**	**0.742**	-0.046	0.038	0.005	-0.043	-0.019	0.133	0.125	-0.062	-0.040
M8	0.637	0.627	0.585	0.003	1.000	0.009	0.175	0.655	**0.713**	0.180	0.555	0.195	-0.019	0.343	**0.858**	**0.768**	**0.771**	**0.742**	0.561	0.433	0.452	0.226
M9	0.582	0.199	0.192	0.109	**0.809**	1.000	0.263	0.661	**0.928**	0.103	0.402	0.137	0.097	0.499	0.702	0.534	0.661	0.576	0.110	0.367	0.110	0.295
M11	0.402	0.220	0.286	-0.061	0.175	0.263	1.000	**0.705**	0.285	**0.800**	0.488	-0.048	-0.053	0.373	0.339	0.103	0.201	0.329	-0.052	0.486	0.317	0.313
M13	**0.707**	0.410	0.495	0.140	0.655	0.661	**0.759**	1.000	0.555	**0.705**	0.659	0.177	0.100	0.295	0.672	0.437	0.457	0.619	0.237	0.625	0.331	0.175
M14	0.603	0.190	0.104	0.013	**0.713**	**0.925**	0.285	0.555	1.000	0.072	0.425	0.051	0.035	0.688	0.688	0.503	**0.718**	0.539	0.022	0.353	0.197	0.481
M15	0.302	0.304	0.570	-0.040	0.180	0.103	**0.860**	**0.705**	0.072	1.000	0.393	0.110	-0.001	0.229	0.233	0.229	0.239	0.144	0.247	0.271	0.257	0.258
M17	**0.769**	0.575	0.533	0.001	0.555	0.402	0.488	0.659	0.425	0.393	1.000	0.228	-0.023	0.266	0.692	0.473	0.433	0.663	0.330	0.612	0.584	0.172
M22	0.344	0.255	0.473	**0.737**	0.195	0.137	-0.048	0.177	0.051	0.110	0.228	1.000	0.516	0.053	0.271	0.297	0.194	0.116	0.473	0.135	0.174	0.167
M28	0.236	-0.042	-0.006	**0.742**	-0.019	0.097	-0.053	0.100	0.035	-0.001	-0.023	0.516	1.000	-0.048	-0.012	0.031	-0.045	-0.017	-0.045	0.135	-0.040	-0.058
M31	0.284	0.296	0.137	-0.046	0.343	0.499	0.373	0.295	0.688	0.229	0.266	0.053	-0.048	1.000	0.494	0.370	0.675	0.282	0.086	0.167	0.376	**0.888**
M32	**0.785**	**0.783**	0.514	0.038	**0.858**	**0.702**	0.339	0.672	0.688	0.233	0.692	0.271	-0.012	0.494	1.000	0.680	0.677	**0.716**	0.413	**0.739**	**0.715**	0.415
M35	0.511	0.700	**0.748**	0.005	**0.768**	0.534	0.103	0.437	0.503	0.229	0.473	0.297	0.031	0.370	0.680	1.000	**0.779**	0.542	0.692	0.203	0.561	0.408
M39	0.459	0.552	0.584	-0.043	**0.771**	0.661	0.201	0.457	**0.718**	0.239	0.433	0.194	-0.045	0.675	0.677	**0.779**	1.000	0.485	0.562	0.160	0.467	0.617
M40	**0.753**	**0.749**	0.360	-0.019	**0.741**	0.576	0.329	0.619	0.539	0.144	0.663	0.116	-0.017	0.282	**0.916**	0.542	0.485	1.000	0.243	**0.860**	**0.736**	0.240
M41	0.220	0.664	**0.871**	0.133	0.561	0.110	-0.052	0.237	0.022	0.247	0.330	0.473	-0.045	0.086	0.413	0.692	0.562	0.243	1.000	-0.029	0.471	0.188
M43	**0.775**	0.570	0.149	0.125	0.433	0.367	0.486	0.625	0.353	0.271	0.612	0.135	0.135	0.167	**0.739**	0.203	0.160	**0.860**	-0.029	1.000	0.615	0.158
M45	0.542	**0.906**	0.556	-0.062	0.452	0.110	0.317	0.331	0.197	0.257	0.584	0.174	-0.040	0.376	**0.715**	0.561	0.467	**0.736**	0.471	0.615	1.000	0.458
M46	0.222	0.374	0.218	-0.040	0.226	0.295	0.313	0.175	0.481	0.258	0.172	0.167	-0.058	**0.888**	0.415	0.408	0.617	0.240	0.188	0.158	0.458	1.000

Note:

[1]M3 = Hexadecane; M4 = 1-iodo-2-methylundecane; M5 = Pentadecane; M6 = Undecane; M8 = 2,4,6-trimethyl octane; M9 = 4-methyl-1-undecene; M11 = 1-iodoundecane; M13 = Eicosane; M14 = 2 methyl heptadecane; M15 = Allyl nonyl ester oxalic acid; M17 = 2,4-bis(1,1-dimethylethyl) phenol; M22 = 2,4-dimethyl undecane; M28 = 2,3,3-trimethyl pentane; M31 = 2-methyl dodecane; M32 = 2,3,5,8-tetramethyl decane; M35 = 3,3-dimethyl hexane; M39 = 2,6,10,14-tetramethyl heptadecane; M40 = 2-benzoyl methyl ester benzoic acid; M41 = Allyl octyl ester oxalic acid; M43 = Tetradecane; M45 = 2,9-dimethyl decane; and M46 = 1-iodo-tetradecane.

[2] $|0.000| < R < |0.300|$ = weak correlation, $|0.300| < R < |0.700|$ = moderate correlation and $|0.700| < R < |1.000|$ = strong correlation matrix.

[3]Correlation matrix with the bold value indicated a strong correlation between two metabolites

From Table 3, 2,3,5,8-tetramethyl decane had 7 strong CMs followed by 2,4,6-trimethyl octane and 2-benzoyl methyl ester benzoic acid (6 CMs), hexadecane (3 CMs), and 1-iodo-2-methylundecane (4 CMs). Also, pentadecane, 4-methyl-1-undecene, eicosane, 2-methyl heptadecane, 3,3-dimethyl hexane, 2,6,10,14-tetramethyl heptadecane, tetradecane, and 2,9-dimethyl decane individually had 3 CMs. Among these metabolites, the 2,3,5,8-tetramethyl decane had strong correlation with 2-benzoyl methyl ester benzoic acid (R = 0.916), 2,4,6-trimethyl octane (R = 0.858), hexadecane (R = 0.785), 1-iodo-2-methylundecane (R = 0.783), tetradecane (R = 0.739), 2,9-dimethyl decane (R = 0.715) and 4-methyl-1-undecene (R = 0.701). Although these metabolites rendered strong CM, the CM value only explained the correlation between two metabolites [20] while the CMs among more than two metabolites bring more meaningful information, where the PCA can cater to this purpose [21].

3.2 Metabolite correlations

The Pearson correlation test was chosen to select suitable correlation types to determine correlations between two metabolites. The correlation explores the dataset in investigating the strength (weak, moderate, or strong) and direction (positive and negative) of the linear relationship between two metabolites. Table 2 shows the correlation matrix (CM) of the metabolites in the urine of dengue-infected patients. The CM measures the strength and direction of a linear relationship between two metabolites. The CM indicated that positive and negative correlations among the metabolites at $|0.700| < R < |1.000|$ for strong CM, $|0.300| < R < |0.700|$ for moderate CM, and $3|0.000| < R < |0.300|$ for weak CM as in generic guideline. From Table 3, 2,3,5,8-tetramethyl decane had 7 strong CMs followed by 2,4,6-trimethyl octane and 2-benzoyl methyl ester benzoic acid (6 CMs), hexadecane (3 CMs), and 1-iodo-2-methylundecane (4 CMs). Also, pentadecane, 4-methyl-1-undecene, eicosane, 2-methyl heptadecane, 3,3-dimethyl hexane, 2,6,10,14-tetramethyl heptadecane, tetradecane and 2,9-dimethyl decane individually had 3 CMs. Among these metabolites, the 2,3,5,8-tetramethyl decane had strong correlation with 2-benzoyl methyl ester benzoic acid (R = 0.916), 2,4,6-trimethyl octane (R = 0.858), hexadecane (R = 0.785), 1-iodo-2-methylundecane (R = 0.783), tetradecane (R = 0.739), 2,9-dimethyl decane (R = 0.715) and 4-methyl-1-undecene (R = 0.701). Although these metabolites rendered strong CM, the CM value only explained the correlation between two metabolites [20] while the CMs among more than two metabolites bring more meaningful information, where the PCA can cater to this purpose [21].

Table 3. Metabolites with strong and moderate factor loading
in the urine of dengue-infected patients

Metabolite	Designation	Factor loading (FL)[1,2]		
		F1	F2	F3
Hexadecane	M3	**0.8415[a]**	0.1805	-0.0806
1-iodo-2-methylundecane	M4	**0.7572[a]**	-0.1588	0.4861
Pentadecane	M5	**0.6556[b]**	0.0136	**0.6144[b]**
Undecane	M6	0.2016	**0.8113[a]**	-0.1352
Heptacosane	M7	**0.5897[b]**	**0.5315[b]**	-0.1862
2,4,6-trimethyl octane	M8	**0.8207[a]**	-0.2194	0.1018
4-methyl-1-undecene	M9	**0.6737[b]**	-0.1698	-0.4236
Eicosane	M13	**0.7683[a]**	0.0420	-0.1401
2,4-bis(1,1-dimethylethyl) phenol	M17	**0.7467[b]**	-0.0005	0.1296
Dodecamethyl cyclohexasiloxane	M20	0.1414	**0.7147[b]**	-0.1438
2,4-dimethyl undecane	M22	0.4259	**0.6404[b]**	0.1520
2,3,3-trimethyl pentane	M28	0.1722	**0.8061[a]**	-0.2255
2-bromooctane	M29	**0.6308[b]**	0.4901	0.2031
2-methyl dodecane	M31	**0.5367[b]**	-0.4156	-0.4666
2,3,5,8-tetramethyl decane	M32	**0.9265[a]**	-0.1847	0.0067
3,3-dimethyl hexane	M35	**0.7333[b]**	-0.1918	0.3373
Hexyl octyl ester sulfurous acid	M38	0.3339	**0.5490[b]**	0.1871
2,6,10,14-tetramethyl heptadecane	M39	**0.7423[b]**	-0.3650	0.0196
2-benzoyl methyl ester benzoic acid	M40	**0.8040[a]**	-0.1474	0.0605
Tetradecane	M43	**0.6782[b]**	0.1122	-0.1129
2,9-dimethyl decane	M45	**0.6970[b]**	-0.1692	0.3435
Eigenvalue		11.4543	4.0302	3.1970
Variability, %		24.9006	8.7614	6.9500
Cumulative explained variability (CEV), %		24.9006	33.6619	40.6120

Note:
[1]a FL $\geq |0.750|$ = strong factor loading and b $|0.500| <$ FL $< |0.749|$ = moderate factor loading in the principal component.
[2] Factor loading with bold superscript a indicated strong and moderate factor loading in the principal component.

3.3 Dataset exploration by principal component analysis

The purpose of using PCA in this study was to investigate the metabolite contribution to the urines and identify the variance of intercorrelated metabolites when patients were infected with the dengue virus.

Fig. 1 (a) shows the correlation biplot between the metabolites and urines which explains the metabolite contribution to the individual urine samples. The F1 and F2 axes explained 8.76% and 24.90% of the dataset respectively, which cumulatively explained 33.66% of the total dataset. From the correlation biplot in Fig. 1 (a), C1, C2 and C3 replicate i.e control urines of dengue-free patients were located in the F1 < 0 axes only. Also, D1, D2, D3, and D5 belonged together with these control urines. Fig. 1 (b) depicted that hexatriacontane, 2-ethylhexyl hexyl ester sulfurous acid, dotriacontane, hexyl pentyl ester sulfurous acid and 3-isopropoxy-1,1,1,7,7,7-hexamethyl-3,5,5-tris(trimethylsiloxy)tetrasiloxane were dominant in five control urines: C1-4, C2-3, C2-4, C3-2 and C3-5. Meanwhile, 2,2,3,3,5,6,6-heptamethyl heptane, 4,4-trimethyl hexane, 2,3,5,8-tetramethyl decane, and N,N,O-triacetylhydroxylamine were dominant in another 10 replicates of the control urines.
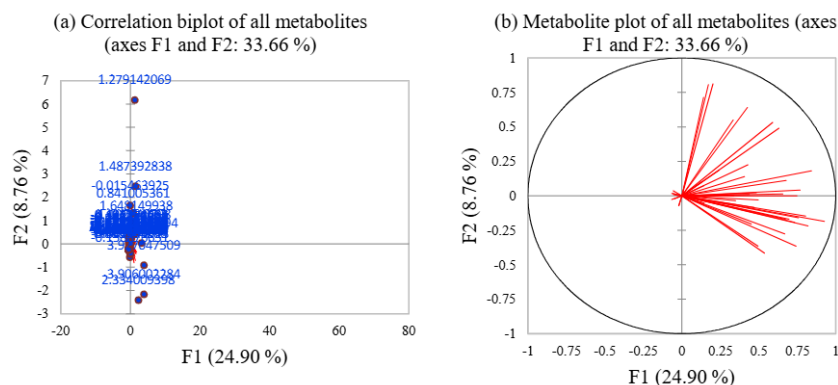
Figure 1. (a) Metabolite plot and (b) correlation biplot of all metabolites in
the urine of dengue-infected patients

Only the urines of dengue-infected patients were present at $F1 > 0$, while no urine controls fell to this side (Fig. 1(a)). Moreover, 37 metabolites contributed to this site and since the urines present in this site are from the dengue-infected patients (Fig. 1(b)), this study proposed these metabolites to be the potential biomarkers in urine to discriminate the dengue-infected patients from the healthy ones. However, Daddiouaissa et al.(2021) proposed the selection of significant metabolites as biomarkers based on the calculated factor loading (FL) in the PCA; hence, this study selected and discussed metabolites with strong ($FL \geq |0.750|$) and moderate ($|0.500| < FL < |0.749|$) only.

Table 3 exhibited the PCA with three PCs for the urine metabolites consisting of 21 significant metabolites ($p < 0.05$), respectively. Since the PCA is a two-dimensional PC, only PC1 and PC2 had cumulative explained variability (CEV) of 33.66% with an eigenvalue (EV) of 4.03 for the whole dataset. Within these PCs, 15 metabolites have a simultaneous and positive correlation among them in PC1, while 6 metabolites had simultaneous and positive correlation among them in PC2. Among them, hexadecane, 1-iodo-2-methylundecane, 2,4,6-trimethyl octane, eicosane, 2,3,5,8-tetramethyl decane, and 2-benzoyl methyl ester benzoic acid had strong factor loading (FL) in PC1 while the undecane and 2,3,3-trimethyl pentane had strong FL in PC2 ($FL \geq |0.750|$), indicating that these metabolites were dominant in the urine of dengue-infected patients due to two individual potential factors.

The pentadecane, heptacosane, 4-methyl-1-undecene, 2,4-bis(1,1-dimethylethyl) phenol, 2-bromo octane, 2-methyl dodecane, 3,3-dimethyl hexane, 2,6,10,14-tetramethyl heptadecane, tetradecane and 2,9-dimethyl decane had moderate FL in PC1 while heptacosane, dodecamethyl cyclohexasiloxane, 2,4-dimethyl undecane and hexyl octyl ester sulfurous acid had moderate FL in PC2 ($|0.500| < FL < |0.749|$).

3.4 Discriminant of urines of dengue-infected patients and control

Table 1 shows the classification matrix of training and cross-validation datasets of urine of dengue-infected patients and controls by discriminant analysis. In this study, the establishment of

discriminating model 1 (DM1) applied metabolites with strong and moderate factor loading from the PCA in the training dataset which rendered 90.91% and 100.00% correct classification for urines of dengue-infected patient and controls, respectively. The average of correct classification was 96.15% for a total of 26 urines. The correct classification < 100% was due to DM1 employing 21 metabolites entailing strong and moderate FLs, whereas the metabolites with moderate FL may have reduced the classification ability of DM1. Moreover, the location of D7-4 and D9-3 urines from the PCA, which was nearer to the cluster of urine controls, contributed to 96.15% correct classification. These reasons were supported by the location of both cluster centroids, as depicted in Fig. 2(a), which indirectly reduced the correct classification of the cross-validation dataset to 76.92% for the same number of total urines. The DM1 provided information on which metabolites were the most significant ones via F-statistic value and p-value. Since the DM1 was established at α of 0.05; therefore, selecting metabolites with F-statistic < 5 and p-value < 0.05, and removing D7-4 and D9-3 urines from the DM2 in Table 4 shows the 100% correct classification of the training dataset for the dengue-infected patient, controls, and average classification for a total of 24 urines. This result indicated that employing metabolites with F-statistic < 5 and p-value < 0.05, and urines except D7-4 and D9-3 had improved the correct classification of urines of dengue-infected patients and controls. This was evident in Fig. 2(b), which depicted the cluster centroids of dengue-infected patients and controls were further separated as compared to although C2-4 fell beyond the hoteling border. Additionally, correct classification for cross-validation in DM2 had also improved to 87.50% by using the same treatment as compared to DM1.
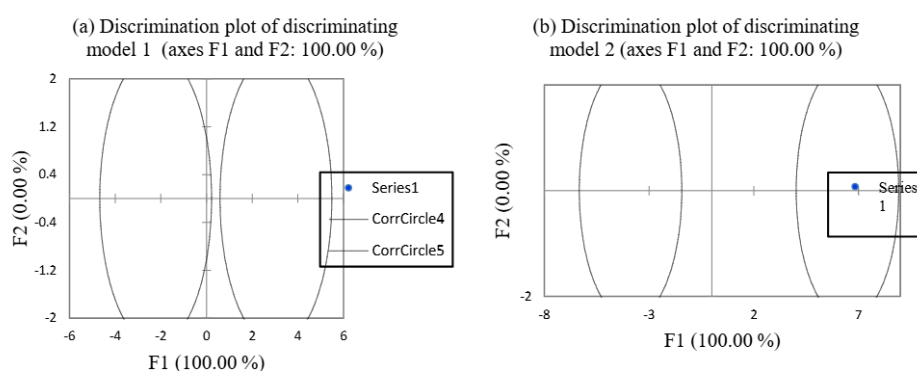

Figure 2. Discrimination plot of (a) discriminating model 1 and (b) discriminating model 2

Based on the F-statistic < 5 and p-value < 0.05, the DM2 had selected nine significant metabolites that could be proposed as potential biomarkers to assist in identifying dengue-infected patients. The nine metabolites' significance rankings were as follows: Heptacosane > Hexadecane > 2,4-bis(1,1-dimethylethyl) phenol > 2-bromooctane > tetradecane > hexyl octyl ester sulfurous acid > 2-benzoyl methyl ester benzoic acid > 2,9-dimethyl decane > pentadecane. Among these metabolites, six of them were alkane, one phenol, and two acid groups. Bouatra and co-workers[22] found the presence of 2,4-bis(1,1-dimethylethyl) phenol and benzoic acid group in a pool of human urine; however, they did not associate the presence of this metabolite with the dengue-infected patient.

Table 4. Classification matrix of training and cross-validation datasets of urine of dengue-infected patients and controls by discriminant analysis

Discriminating model (DM)	Treatment	Dataset	Correct classification, %	Number of urines and p-values of Fisher distance[1]		Total urine	Ranking of significant metabolites contributed to the DM ($p < 0.05$)[2]
				The urine of a dengue-infected patient	Urine of control		
Discriminating model 1 (DM1)	Application of metabolites with strong and moderate factor loading from the PCA	Training dataset					Hexadecane (33.5959,) > Heptacosane (22.5566) > 2-bromooctane (12.0689) > Tetradecane (11.2645) > 2,4-bis(1,1-dimethylethyl) phenol (10.8625) > 2-benzoyl methyl ester benzoic acid (8.2477) > Hexyl octyl ester sulfurous acid (6.9570) > 2,9-dimethyl decane (6.1448) > Pentadecane (5.4461)
		The urine of a dengue-infected patient	90.91	10 (1)	1 (< 0.0011)	11	
		Urine of control	100.00	0 (< 0.0011)	15 (1)	15	
		Average	96.15	10	16	26	
		Cross-validation dataset					
		The urine of a dengue-infected patient	54.55	6 (1)	5 (< 0.0011)	11	
		Urine of control	93.33	1 (< 0.0011)	14 (1)	15	
		Average	76.92	7	19	26	
Training dataset / The urine of a dengue-infected patient / Urine of control / Average / Cross-validation dataset / The urine of a dengue-infected patient / Urine of control / Average	(1) Application of metabolites with (a) strong and moderate factor loading from the PCA and (b) F-statistic < 5 and p-value < 0.05 only. (2) Removal of urine sample D7-4 and D9-3 / 100.00 / 100.00 / 100.00 / 66.67 / 100.00 / 87.50	Training dataset					Heptacosane (41.1045, < 0.0001) > Hexadecane (35.4330, < 0.0001) > 2,4-bis(1,1-dimethylethyl) phenol (13.6746, 0.0013) > 2-bromooctane (11.9207, 0.0023) > Tetradecane (10.6397, 0.0036) > Hexyl octyl ester sulfurous acid (9.5052, 0.0054) > 2-benzoyl methyl ester benzoic acid (8.9872, 0.0066) > 2,9-dimethyl decane (8.2742, 0.0088) > Pentadecane (6.7105, 0.0167)
		The urine of a dengue-infected patient	100.00	9 (1)	0 (< 0.0011)	9	
		Urine of control	100.00	0 (< 0.0011)	15 (1)	15	
		Average	100.00	9	15	24	
		Cross-validation dataset					
			66.67	6 (1)	3 (< 0.0011)	9	
			100.00	0 (< 0.0011)	15 (1)	15	
			87.50	6	18	24	

Note:
[1] Calculated p-value of Fisher distance < 0.05 indicated two clusters were significant difference.
[2] Values in parenthesis were F-statistic value and p-value

The production of alkane in the dengue-infected patient suggests that the host cells are experiencing oxidative stress as mentioned by Gil and co-workers [23], which then leads to lipid peroxidation [24] and alkane production [25]. Alkane produced was then secreted by the host through urine supported by Smith and co-workers [26] when they identified more than 200 volatiles in human urine, which belonged to different chemical classes such as alcohols, ketones, hydrocarbons, pyrroles, furans, aldehydes, terpenes, sulfur-containing compounds (isocyanates, sulfides), and O- and N-heterocyclic compounds. Courageot and co-workers [27] reported that the dengue (DEN) virus can trigger the host cells to undergo apoptosis in a cell-dependent manner and it is confirmed by Di Meo and co-workers[28] that dengue viruses caused apoptosis through lipid peroxidation. Suggesting that the alkane identified in our study is a result of dengue virus infection.

The 2,4-bis(1,1-dimethylethyl) phenol is a common toxic secondary metabolite produced by organisms and functions in ensuring endocidal regulation [29]. Moreover, Leila and co-workers[30] reported that the 2,4-bis(1,1-dimethylethyl) phenol exhibited significant anti-coxsackievirus B-3 (CVB-3) and anti-herpes virus type 2 (HSV-2) activities. Both studies supported the presence of a high amount of 2,4-bis(1,1-dimethylethyl) phenol in this study. Furthermore, Brynildsen and co-workers[31] through their study found that 2,4-bis(1,1-dimethylethyl) phenol also increased microbial ROS production that can potentiate killing the parasites.

4. CONCLUSION

This study shows that the GCMS approach can identify metabolite biomarkers from the urine of dengue patients. Heptacosane, Hexadecane, 2,4-bis(1,1-dimethylethyl) phenol, 2-bromooctane, tetradecane, hexyl octyl ester sulfurous acid, 2-benzoyl methyl ester benzoic acid, 2,9-dimethyl decane, and pentadecane are the potential biomarkers for dengue virus detection using a human urine sample.

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