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The Evaluation on Molecular Techniques of Reverse Transcription Loop-Mediated Isothermal Amplification (RT-LAMP), Reverse Transcription Polymerase Chain Reaction (RT-PCR), and Their Diagnostic Results on MinION™ Nanopore Sequencer for the Detection of Dengue Virus Serotypes

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Abstract **Background and aims**:. Reverse Transcription Loop-Mediated Isothermal Amplification (RT-LAMP) Assay is a method described as a simple, rapid, and cost-effective, and a powerful gene amplification technique for rapid identification of microbial infection. The aims of this study were to validate a reverse-transcription LAMP method (RT-LAMP) with a reverse-transcription PCR technique (RT-PCR) for DENV serotype detection, and to evaluate the potential of this RT-LAMP method from which its amplification product can be applied for MinIONTM Nanopore sequencing. **Methods:** This study was performed by using 26 patient serum samples from the Confirmed group and 23 sera from the Suspected group, and 10 sera from Healthy volunteer group. The same validation study to confirm the sensitivity and specificity between the RT-LAMP assay and RT-PCR was also conducted by making a serial dilution of template as well as dengue serotype detected sera. Furthermore, experiments of MinIONTM Nanopore sequencer using the amplification product (amplicon) of RT-LAMP assay from patient serum samples and its comparative diagnostics with RT-PCR and Sanger's Sequencing were also done. **Results:** From the validation study, 17 (65.4%) out of the 26 sera of Confirmed group were serotype detected by RT-LAMP assay, compared to that of RT-PCR which was only 10 (38.4%). Same result in Suspected group, there were 11 (47.8%) out of 23 sera of Suspected group were serotype detected by RT-LAMP assay, compared to that of RT-PCR which was only 1 (4.3%). For the Healthy group, the result was somewhat comparable between the two methods. In the serial dilution study, RT-LAMP was superior than that of the RT-PCR technique, particularly seen in the serial dilution of clinical serum samples. The limit of detection of RT-LAMP was down to the titer of 1/1000x, while RT-PCR was only 1/10x. Secondly, amplicon of RT-LAMP assay from patient serum samples fits to be sequenced by the MinIONTM. In the comparative study, the results showed that RT-LAMP was more sensitive and specific compared to that of the RT-PCR technique and Sanger's Sequencing. **Conclusions**: RT-LAMP assay was more sensitive to that of RT-PCR technique. In the comparative study using MinIONTM Nanopore sequencer for the detection of dengue virus serotypes, the RT-LAMP was more sensitive and specific to that of RT-PCR and Sanger's Sequencing. The method of RT-LAMP should be applied more because its sensitive and specific, simple, rapid, cost-effective, and also can be a tool of point of care testing for the detection of dengue virus serotypes**.**

Keywords: *dengue virus serotypes, reverse transcription loop-mediated isothermal amplification assay (RT-LAMP)*

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1. Introduction

Dengue virus infection has been a continuing global health problem mainly in Southeast Asia, in where Indonesia is the most populated country in this tropical developing region. Epidemics of dengue fever (DF) and dengue hemorrhagic fever (DHF) have become a serious health problem through Indonesian archipelago since the incidence rate and case fatality rate have been erratically increased every year. [1] WHO 2011, recorded that Indonesia has the highest incidence rate of dengue hemorrhagic fever (DHF) ever since 1968 until 2009. [2] Dengue viruses has four antigenically distinct serotypes, namely Den-1, Den-2, Den-3, Den-4, respectively. [3,4] The four serotypes of dengue virus share a common morphology and genomic structure as well as common antigenic determinants with members of the genus Flavivirus, family Flaviviridae, consisting of over 60 arthropod-borne viruses. [3] A mature dengue virion is relatively small, 40-50nm, consisting of a single-stranded, positive sense RNA genome, surrounded by a nucleocapsid, and a membrane with a lipid envelope. [3,4] The dengue virus genome is approximately 11.000 bases long and is made up of three structural (capsid, membrane, envelope) and seven nonstructural proteins (NS1, NS2a, NS2b, NS3, NS4a, NS4b, NS5). [3,4,5] The envelop protein has complex-reactive epitopes which may play a role in the immunopathology of disease [3,4,5]. Infection at the first time with any of the serotypes (primary infection) leads to a mild form of illness such as self-limiting fever, however in secondary infection a more severe form of disease, involving vascular and hemostatic abnormalities, DHF, and dengue shock syndrome (DSS), is responsible for a high case fatality rate, especially in children. [4,5] It is believed that antibody arose after primary infection can lead a life-long protective immunity to the homologous serotype but not in the secondary infection. The existence of weak neutralizing antibodies to one serotype may promote the enhancement of infection upon the next infection with another serotype. In the hyperendemic region there are more than one serotype circulate leading to multiple and sequential infections with the four dengue virus serotype due to the lack of cross-protective neutralizing antibodies. Therefore, rapid detection and differentiation between primary and secondary dengue virus infections and determination of the dengue virus serotypes of past and present infections are important for patient management and epidemiological studies. Reverse Transcription Loop-Mediated Isothermal Amplification (RT-LAMP) Assay is a method described as a simple, rapid, and cost-effective, and a powerful gene amplification technique for rapid identification of microbial infection and also is of particular interest for field diagnosis of tropical disease.

The aims of this study is to evaluate the RT-LAMP assay for use for clinical diagnosis with a number of patient serum samples, to compare with that of RT-PCR for its sensitivity, as well as the use of virus template and patient serum samples in the serial dilution experiment. The experiment using MinION^{TM} to sequence the amplicon of RT-LAMP assay and to compare with that of Sanger's sequencing and RT-PCR was also carried out to see its sensitivity and specificity.

2. Materials and Methods

2.1. Clinical Samples

A total of 158 collected sera of patient serum samples, obtained from May 2014 until February 2015, from Manado (mostly from patients of Prof. dr. R.D. Kandou General Provincial Hospital) and adjoining areas (Minahasa) were included in the study after approval of hospital ethical committee. The study work was conducted in Laboratorium Pro-Kita Manado which is located nearby and also has a collaboration for serum sample collection with Prof. dr. R.D. Kandou General Provincial Hospital, Biotechnology Laboratory of Faculty of Mathematics and Physics of Sam Ratulangi University (UNSRAT) Manado, for additional study work, and two separate laboratories in Japan for results confirmation. One is from the Department of Computational Biology, the University of Tokyo (Prof. Yutaka Suzuki), the other is from the Department of Infectious Disease Control, Faculty of Medicine, Oita University (Prof. Yuki Eshita). Cases of all age groups were selected using WHO guidelines 2011 for dengue virus infection. There were 158 collected sera that were divided into 2 groups, namely, Confirmed (NS-1 positive) including 80 sera and Suspected (with no NS-1 data) 78 sera for this study. **Firstly**, validation study for RT-LAMP assay with the technique of conventional RT-PCR as a standard method for dengue virus serotype detection was carried out. This study was performed by using 26 patient sera from the Confirmed group and 23 sera from the Suspected group, and 10 sera from Healthy volunteer group. The same validation study to confirm the sensitivity and specificity between the RT-LAMP assay and RT-PCR was also conducted by making a serial dilution of template and of dengue serotype detected sera, DENV1,-2,-3,-4, respectively. **Secondly**, experiments of MinION^{TM} Nanopore sequencer using the amplification product (amplicon) of RT-LAMP assay from patient sera, and its comparative evaluation on the results between RT-PCR and Sanger's Sequencing were also done.

2.2. Cell Culture

C6/36 mosquito cell was cultured in 10% MEM medium (10% FBS, 1% Non-Essential Amino Acid solution, Invitrogen; 1% Glutamax, Invitrogen) by a disposable flask.When the cell titer becomes 80-90% confluent, medium was changed to 2% MEM. Remove the medium before the viral infection.100microl of Dengue virus seed (virus titer = $2x10^7$ PFU/mL; PFU=Plaque Forming Unit) was added. Incubate at 28^0C under 5% CO2 for 90 min. Add 10 mL of 2% MEM10. Incubate at 28^0C for five days. Harvest the supernatant. Centrifuge it at 2500rpm for 10 min. Store at – 80^0C

2.3. RNA Extraction

Scale up protocol for virus suspension:

[Combination of Trizol-LS reagent (Invitrogen) and RNAeasy Mini Kit (Qiagen)]

Trizol-LS reagent: Keep at 4℃, Invitrogen

RNAeasy Mini Kit: Keep at RT, For 20 samples, Cat.#74103, Qiagen (RNA extraction, according to the manufacturer's protocol)

2.4. RT-LAMP Primers

Universal primers: **DEN-1** 10463-10672, FIP: TACAGCTTCCCCTGGTGTTTTGTGGTTAGAGGAG A CCCCT, BIP: AGAGGTTAGAGGAGACCCCCTT TTGAGACAGCAGGATCTCTGGT, LB: AGCATAT TGACGCTGGGAGAG, F3: GGAAGCTGTACGC ATGG, B3: CTGTGCCTGGAATGATG.

For Serotype1: DEN-1 10469–10667, FIP: GCTGCGTTGTGTCTTGGGAGGTTTTCTGTACGCAT GGG GTAGC, BIP: CCCAACACCAGGGGAAGCTG TTTTTTTGTTGTTGTGCGGGGG, FLP: CTCCTCTAA CCACTAGTC, BLP: GGTGGTAAGGACTAGAGG, F3: GAGGCTGCAAACCATGGAA, B3: CAGCAG GATC TCTGGTCTCT. **For Serotype2**: DEN-2 10449–10659, FIP: TTGGGCCCCCATTGTTGCTGTTTT AGTGGA CTAGCGGTTAGAGG, BIP: GGTTAGAGGAGACCCC CCCAATTTTGGAGACAGCAGGA TCTCTGG, FLP: GATCTGTAAGGGAGGGG, BLP: GCATATTGA CGCTGGGA, F3: TGGAAGCTGT ACGCATGG, B3: GTGCCTGGAATGATGCTG. **For Serotype3**: DEN-3 10289–10506, FIP: TGGCTT TTGGGCCTGACTTCTTT TTTGAAGAAGCTGTGCAGCCTG, BIP: CTGTAGC TCCGTCGTGGGGATTTTCTAGTCTGCTACACCGTG C, FLP: CCTTGGACGGGGCT, BLP: GGAGGCTG CAAACCGTG, F3: GCCACCTTAAGCCACAGTA, B3: GTTGTGTCATGGGAGGG. **For Serotype4**: DEN-4 10289–10517, FIP: TGGGAATTATAACGCCTCCCG TTTTTTCCACGGCTTGAGCAAACC, BIP: GGTTAGA GGAGACCCCTCCCTTTTAGCTTCCTCCTGGCTTCG, FLP: GGCGGAGCTACAGGCAG, BLP: TCA CCAACA AAACGCAG, F3: CTATTGAAGTCAGGCCAC, B3: CTATTGAAGTCAGGCCAC. Retrieved from GenBank and performed using ClustalW (http://clustalw.ddbj.nig.ac.jp).

2.5. RT-LAMP Protocol

Prepare primer mix: FIP 40 pmol/uL, BIP 40 pmol/uL, FLP 20 pmol/uL, BLP 20 pmol/uL, F3 5p pmol/uL, B3 5p pmol/uL, Total 1 uL each/sample.

Prepare buffer mix: 0.15 TritonX 9.97uL, CFI 1, RNasin 0.1, Bst HS polymerase 1, AMV rt 0.03, 25x LAMP buffer 1, 100 mM MgSO4 1.5, 25mM dNTPs 1.4, 2M Trehalose 218uL/sample.

Procedure: 1. Primer Mix is mixed with Buffer Mix in one single tube, and add RNA Template (1 uL), 2. Incubate at 63^0C for 60 - 90 minutes. 3. Agarose gel analysis. 4. Visualization by the naked eye.

2.6. RT-PCR Primers

Universal primers: DEN-1 132-482, DC1: TCAATATGCTGAAACGCGCGAGAAACCG, DC2: TTGCA CCAACAGTCAATGTCTTCAGGTTC. **For Serotype1**: D1S, GGACTGCGTATGGAGTTTTG, D1C: ATGGGTTGTGGCCTAATCAT, **For Serotype2**: DEN-2 1201-1432, D2S: AGRTTYGTCTGCAAACA CTCC, D2C: GTGTTACTTTRATTTCCTTG, **For Serotype3**: DEN-3 2253-2572, D3S: GTGCTTACACAGC CCTATTT, D3C: TCCATTCTCCCAAGCGCCTG. **For Serotype4**: DEN-4 3976-4375, D4S: CCA TTATGGCT GTGTTGTTT, D4C: CTTCATCCTGCTTCACTTCT.

2.7. RT-PCR Protocol

Preparation of master mix (reaction mix, primer (sense), primer (comp), RT-PCR enzyme mix), template RNA samples. Total volume per tube 12.5uL. Procedures: mix the above master mix thoroughly, transfer 6.875 µl of master mix into 0.2 ml flat-cap PCR tubes on ice, add 5.625 µl template RNA into individual PCR tubes, as for negative control, as for positive control, heat the PCR machine according to these steps: start the machine, on the four corners of tube plates, put the same type of tubes (dome-cap tubes or flat-cap tubes), close and lock the lid using very light power, when resistance is felt, open the lid, turn the lock approximately 60º, and then close lid, set the machine program: choose EDIT, YUKI, RTPCR-D3, check for the correct setting: reverse transcription reaction: 50ºC for 30 minutes, initial PCR activation step: 94ºC for 2 minutes, denaturation: 94ºC for 30 seconds, annealing: 53ºC for 30 seconds, extension: 68ºC (or 72ºC) for 1 minute, amplification: 30 cycles, final extension 68ºC for 2 minutes, 10ºC forever, choose RUN, YUKI, RTPCR-D3, TUBES (not PLATES), type final volume of each tube, and end. Machine will start to heat. Wait until it reaches 50ºC and then pause. Transfer all of the reaction tubes to the plates. Close the lid and resume program.

2.8. The MinION™

LAMP: Reagents: 0.1% Triton-X 9.97 ul, 25x LAMP buffer 1 ul, 100 mM MgSO4 1.5 ul, 25 mM dNTP 1.4 ul, CFI 1 ul, RNAse inhibitor 0.1 ul, BST enzyme 1 ul, AMV reverse transcriptase 0.03 ul 2M, trehalose 2 ul, FIP 40 uM 1 ul, BIP 40 uM 1 ul, FLP 20 uM 1 ul, BLP 20 uM 1 ul, F3 5 uM 1 ul, B3 5 uM 1 ul, Template1 ul. Incubate in 63 degree for 60-90 minutes. Template preparation: Mix sample until 80uL, Because the sample is LAMP, no fragmentation is needed, End-repair: LAMP amplicon 80 uL, NEBNext End Repair 10x buff 10 uL, NEBNext End Repair enzyme 5 uL, DNA CS 5 uL. Incubate in 20 degree for 30 mins. Add 1x Agencourt AMPure 100 uL. Separate pellet from supernatant by putting it in magnetic stand. Remove supernatant. Rinse with 200 uL 80% ethanol 2x. Dry pellet. Add 27 ul nuclease free water and mix. Put on magnetic stand, and transfer 25 ul supernatant to new tube. dA-tailing: NEBNext End Repair 10x buff 3 uL, NEBNext dA-tailing enzyme 2 uL, End-repaired LAMP amplicon 25 uL. Incubate in 37 degree for 30 mins. Ligation with adapters: dA-tailed LAMP amplicon 30 uL. Nuclease free water 8 uL, Adapter mix (AMX) 10 uL, HP adapter (HPA) 2 uL, Blunt/TA ligase master mix 50 uL. Incubate in room temperature for 10 mins. While waiting, wash 10 ul Dynabeads His-tag with wash buffer (WSB). Add 100 ul ligated LAMP amplicon with 100 ul washed Dynabeads His-tag. Remove pellet from supernatant by putting it in magnetic stand. Remove supernatant. Rinse beads with wash buffer 2x. After rinsing, elute the pellet with elution buffer. Add the sample to MinION loading dock with EP buffer and fuel mix. Run the sequencer through MinKNOW control panel.

3. Results

RT-LAMP assay was optimized for the rapid detection of dengue virus. When examined through naked eye, the results of RT-LAMP assay can be seen as a white-yellow precipitate like in the figure-1, as below:

1. Experiments using both RT-LAMP method and RT-PCR for the detection of dengue virus serotype using the same sample source.

The evaluation of DENV RT-LAMP assay with clinical samples was validated with a limited number of patient serum samples infected with each dengue virus serotype. A total of 49 patient serum samples, comprising of 26 samples from Confirmed cases, 23 samples from Suspected cases, and 10 samples from healthy individuals were processed for RNA extraction with the QIAamp viral RNA mini kit and were simultaneously examined by RT-LAMP, RT-PCR for detection of viral RNA, as described above.

For comparative evaluation, the RT-LAMP assay demonstrated higher sensitivity than conventional RT-PCR as shown by Confirmed cases (Table 1), and Suspected cases (Table 2). Whereas, all 10 healthy serum samples were shown with comparable results by both the tests (Table 3).

Table 1. Comparative evaluation between RT-LAMP assay and RT-PCR for the detection of dengue virus serotype using patient serum samples from Confirmed cases

Confirmed (n=26)			Suspected (n=23)		Healthy (n=10)	
SEROTYPES	RT-LAMP	RT-PCR	RT-LAMP	RT-PCR	RT-LAMP	RT-PCR
D1	11	8	11	1	1	1
D2	2	0	0	0	0	0
D3	3	2	0	0	0	0
D4	1	0	0	0	0	0
NS	5	2	7	0	4	0
ND	4	14	5	22	5	9
Total	26	26	23	23	10	10

ND = not detected, NS = signal not significant / serotype inseparable (positive result for more than 1 serotype, true positive or false positive.

From the validation study, there were 17 (65.4%) out of the 26 sera of Confirmed group the dengue serotypes were detected by RT-LAMP assay, compared to that of RT-PCR which was only 10 (38.4%). Same result in Suspected group, there were 11 (47.8%) out of 23 sera of Suspected group, serotypes were detected by RT-LAMP assay, compared to that of RT-PCR which was only 1 (4.3%). For the Healthy group, the result was somewhat comparable between the two methods.

2. Experiments using both RT-LAMP method and RT-PCR for the detection of dengue virus serotype in a serial dilution fashion.

The sensitivity of The RT- LAMP assay for the detection of DENV RNA serotypes was determined by testing 10x serial dilution of template that had previously been quantified through PFU number determination, and of patient serum samples as well that had been serotyped as previously described, with that of conventional RT-PCR.

Table 2. Detection Limit of the RT-LAMP and RT-PCR using virus template with a 10 x serial dilution

TEMPLATE virus control	PRIMER	DILUTION (SERUM)											
		x1	x1/5		x1/10		x1/100		x1/1000		x1/10000		
		L	P	L	P	L	P	L	P	L	P	L	P
D1	D1	O	O	O	O	O	O	O	O	O	O	X	O
D2	D2	O	O	O	O	O	O	O	X	O	X	O	NA
D3	D3	O	O	O	O	O	O	O	O	O	O	X	O
D4	D4	O	O	O	O	O	O	O	O	O	O	O	NA

L = LAMP, P = PCR, O = detected, x = not detected, NA= not analyzed.

Serial dilution were prepared by diluting the template by RNA-ase distilled water. The estimated viral titer of 10 PFU for the respective serotypes are highlighted in yellow. Note that the sensitivy and specificity of RT-LAMP and RT-PCR are almost at the dimilar lever for the *in vitro* control.

Table 3. Detection Limit of the RT-LAMP and RT-PCR using patient's serum with a 10 x *serial dilution*

TEMPLATE	Detected Serotype	DILUTION (SERUM)									
		x		x1/5		x1/10		x1/100		X1/1000	
		LAMP	PCR	LAMP	PCR	LAMP	PCR	LAMP	PCR	LAMP	PCR
10250115	**D1**	**O**	**O**	**O**	**O**	**O**	**O**	**O**	**X**	**O**	**X**
13250115	**D1**	**O**	**O**	**O**	**O**	**O**	**O**	**O**	**X**	**O**	**X**
61230115	**D2**	**O**	**O**	**O**	**X**	**O**	**X**	**O**	**X**	**O**	**X**
40100215	**D2**	**O**	**O**	**O**	**O**	**O**	**O**	**O**	**X**	**O**	**X**
30100215	**D3**	**O**	**O**	**O**	**X**	**X**	**X**	**X**	**X**	**O**	**X**
52100215	**D3**	**O**	**O**	**X**	**X**	**X**	**X**	**X**	**X**	**NA**	**X**
59230115	**D4**	**O**	**O**	**X**	**O**	**X**	**O**	**X**	**X**	**X**	**X**
13210512	**D4**	**O**	**X**	**X**	**X**	**X**	**X**	**X**	**X**	**NA**	**X**

The dilution of patient serum samples was carried out by a serum of healthy volunteer. The RT-LAMP assays were performed by using the diluted serum directly, whereas for the RT-PCR, the RNA was first extracted from the diluted serums and used for the assay. As can be seen from the table, RT-LAMP was more sensitive and specific in a majority of the cases in clinical samples compared to that of the RT-PCR.

3. Experiments using RT-LAMP amplicon to sequence the genome of dengue serotype by The MinION™ Nanopore sequencing.

cDNA product as the result of RT-LAMP reaction (RT-LAMP amplicon) was sequenced in the device of the MinION™ Nanpore Sequencer. The system was optimized and connected to MinKNOW program of INTERNET, following the procedure according to the protocol of the manufacturer (Oxford Nanopore Technologies). Results of the experiment was shown in the Table 4, as follows:

Table 4. Summary of the analysis for the clinical samples by the MinION™ *Nanopore Sequencing*

Sample ID	# of total reads	diagnosed serotype	#of read (mapped to the corresponding serotype (e-10)	%	#of read (mapped to the corresponding serotype (e-5)	%
05	28,105	D1	1,329	100%	6,053	99%
21	2,077	D1	64	100%	338	100%
17	4,700	D1	102	100%	524	99%
06	14,498	D3	0	ND	2	100%
01	2,540	D1	47	100%	350	99%
11	5,016	D1	177	100%	1,123	99%
Ysample	372	D4	6	100%	35	100%

Statistics of the sequences based on which the serotypes were determined are shown. Number of the sequences of total reads, those mapped to the reference genome at the indicated alignment scores are shown in the second, the fourth and the sixth columns, respectively. The frequency of the reads in the total populations for the fourth and sixth columns are shown in the fifth and the seventh columns, respectively. There were 7 samples that can be read by the MinION accordingly. These findings shows that the nanopore sequencing is definitely compatible with that of the RT-LAMP amplicon. When using *in vitro* dengue virus culture as template, results of RT-LAMP, RT-PCR, Sanger's Sequencing, and MinION™ were the same for sensitivity and specificity. However, the sensitivity and specificity of RT-LAMP products used as samples to be analyzed by MinION were shown to be in fact superior compared to that of the RT-PCR and Sanger's sequencing.

Table 5. Comparison between the results of diagnosis between LAMP, RT-PCR, Sanger's sequencing, and MinION sequencing

Clinical Samples	LAMP	RT-PCR	SANGER's sequencing	MinION
05	D1	ND	ND	D1
21	D1	ND	ND	D1
17	D1	ND	ND	D1
06	D3	ND	ND	D3
01	NS	ND	ND	D1
11	D1	ND	ND	D1
Ysample	NS	ND	ND	D4

4. Discussion

There are several difficulties and limitations in the diagnostic of dengue virus infection. [4,5,6] Laboratory confirmation is crucial as the clinical manifestations of the disease are vary. Dengue virus infection causes a spectrum of illness from the indistinguishable or asymptomatic illness which is the most cases; mild fever or dengue fever (DF) of where Indonesia is endemic or in some parts is hyperendemic, this kind of fever is often nonspecific especially in children. It is usually difficult to distinguish from other illnesses found in tropical areas such as malaria, typhoid, measles, rubella. Dengue fever is generally self-limiting and is rarely fatal. However, as fever subsides, characteristic manifestations of plasma leakage or bleeding manifestations such as petechiae or epistaxis appear making accurate clinical diagnosis possible for dengue hemorrhagic fever (DHF) in many cases. [6,7] 2. Clinical or routine laboratory findings are useful to support clinical symptoms of DF or DHF. Relative neutropenia, lymphocytosis, monocytosis, often marked with atypical lymphocytes are associated with DF. Thrombocytopenia is common, but when plate count is less than 100,000/uL and there is an increase of hemoglobin level and hematocrit value relative to baseline, the evidence of vascular leak syndrome occurs. More severely ill with prominent hemorrhagic syndrome, hemoconcentration indicating plasma leakage, is almost always present in classic DHF but is more severe in patients with shock (dengue shock syndrome, DSS). The pathogenesis of DHF and DSS is based mainly into two theories which are not mutually exclusive. First, and the most commonly accepted is theory known as antibody-dependent enhancement (ADE). This theory is believed that after primary infection, the homologous antibody compatible for the firstly introduced dengue virus serotype can not neutralize the heterologous dengue virus serotype upon coming in the secondary dengue infection, but rather facilitate the virus in the form of immune complex to enter the cells of mononuclear cell lineage. The virus replicates within the cells and the cells produce and secrete vasoactive mediators. The other hypothesis is that the dengue virus varies and changes genetically s a result of a natural selection phenomenon. Phenotypic expression caused by genetic changes in the virus genome may contribute to an increase in replication, severity of disease, and transmission potential. [7,8] In Indonesia, in practical point of view, dengue virus infection is based essentially on clinical signs and symptoms with a minimum routine laboratory work-out such as a complete blood count (CBC) only. Rapid diagnostic test for NS1 antigen or anti-dengue antibodies (IgM or IgG) is increasingly in use. However the current situation may still lead to an increase number of misdiagnosed patients since the blood sampling time is not appropriate. It is almost never found to obtain blood samples just in a convalescence phase. Serological tests such as complement fixation (CF), hemagglutination-inhibition (HI), plaque reduction neutralization test

(PRNT) are mainly for research, especially virus isolation and cell culture. Paired sera of acute and convalescent are needed for IgM or IgG seroconversion (rapid-based test or ELISA) to make a confirmed dengue infection. [7,8,9,10] MAC-ELISA or GAC-ELISA is simple, rapid, sensitive and specific for quantitatively measure the levels of antibodies in serum of dengue infected patients [8,9,10] but it is less demanding particularly in suburban or rural areas. However, confirmed dengue infection can also be achieved by performing rapid diagnostic test for NS1 antigen. [10,11] Rapid diagnostic test is used to qualitatively detect NS1 antigen or anti-dengue IgM/IgG antibodies. It is becoming a routine diagnostic test for dengue virus infection. The NS1 test has gained a lot of interest. Advantages of NS1 tests are NS1 is detected early in disease, several days prior to the appearance of anti-dengue IgM antibodies. It shows a positive correlation with disease severity in secondary infection. [10] The test is less expensive, easy, and fast [11].

In our study work, all NS1 positive sera were categorized in the Confirmed group, whereas the sera from patients who were hospitalized just based on clinical assessment, or having positive for rapid anti-dengue antibodies were all categorized as Suspected sera. Genome detection is one of the diagnostic efforts to confirm dengue infection. Reverse transcription polymerase chain reaction (RT-PCR) has long been designed by Lanciotti et al. 1992 for the use in the diagnosis for dengue genome and the detection of dengue serotypes. [12] Until now this type of nucleic acid amplification technique has been adapted and modified according to recently applied technologies. [13,14,15] However, still the settings of PCR laboratory and the necessity of well trained personnel remain in highly prerequisite. We developed an optimized RT-LAMP method, invented by Notomi et al. 2000 [16,17,18], and validate the method with that of the RT-PCR. 19,20,21] What we found that the RT-LAMP was more sensitive and specific than that of RT-PCR. Our findings can be seen in the results when comparing the both methods for the examination of clinical patient samples, as well as in the serial dilution using either dengue virus control as the template or patient serum samples. This result was in accordance with other findings (Parida, 2005 and Dauner, 2011). [22,23] However, there are numbers of patient serum samples which their serotypes are not detected (ND) by both RT-LAMP and RT-PCR methods. Reasons to these negative findings were supposed to be the appropriate temperature and time length in keeping the serum samples. We used refrigerators that have capacity only to keep sera in the range between - 20^0 to -25^0 Celsius. The serum should be kept in -80 or less after one month drawn, [2] our stored serum samples were mostly kept between 1-6 months. Serum must be obtained as early as possible in the acute phase of illness, making sure that the viremia still in place. [4,5] Another thing to consider is that in secondary infection the virus may be complexed by antibody, making it undetectable [8,10].

In addition to the evaluation comparison between RT-LAMP and RT-PCR methods, we also determined to sequence the amplicon of RT-LAMP in the MinON™ device. It is shown in Table 4, the capability of the MinION™ in the determination of dengue serotype. The MinON™ is a nanopore sequencing technique that was recently launched for use by researchers in early access program (begun at Fall season 2014). [24] A promising of a third generation DNA sequencers which uses nanopore technology awaits for its implementation as a better genome sequencing technology may come into reality since it is portable, cheap, and fast. [24,25] The comparison of the results of diagnosis between RT-LAMP, RT-PCR, Sanger's sequencing, and MinION™ sequencing as shown in Table 5, reveals that the RT-LAMP assay is more sensitive and specific. RT-LAMP can be considered as an ideal dengue genome test.

5. Conclusion

The RT-LAMP assay developed in this study is sensitive, specific, rapid, simple, cost effective with a potential in use along with the MinION™ for its result.

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Conflict of Interest

No conflict of interest for all authors to published this manuscript.

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