Eco. Env. & Cons. 26 (November Suppl. Issue) : 2020; pp. (S1-S5)
Copyright@ EM International
ISSN 0971–765X

Preliminary study of dengue virus serotype on *Aedes* mosquitoes in endemic area, Surabaya, Indonesia, January 2020

Aulia Azzahra[1], Lucky Vera Oktavia[1], Muhammad Fariz Naviyanto[1], Shifa Fauziyah[2], Teguh Hari Sucipto[3a], Dwi Winarni[4], Sri Puji Astutik Wahyuningsih[4], Siti Churrotin[3], Ilham Harlan Amarullah[3] and Soegeng Soegijanto[3]

[1]Department of Biology, Faculty of Sains and Technology, Universitas Airlangga, Surabaya, Indonesia
[2] Master Program of Tropical Medicine, Faculty of Medicine, Universitas Airlangga, Surabaya, Indonesia
[3] Dengue Study Group, Institute of Tropical Disease, Universitas Airlangga, Surabaya, Indonesia
[4] Department of Biology, Faculty of Sains and Technology, Universitas Airlangga, Surabaya, Indonesia

(Received 11 February, 2020; Accepted 28 May, 2020)

ABSTRACT

Aedes sp. is a mosquito that can act as a vector of Dengue Haemorrhagic Fever (DHF). This mosquito can contain dengue virus (DENV) that comes from the blood of dengue sufferers. Dengue virus consists of 4 types of serotypes, namely DENV-1, DENV-2, DENV-3 and DENV-4. In this report, we observe the existence of Dengue virus in *Aedes albopictus* from Institute of Tropical Disease's parking area. Our observation start from mosquito identification, preparation and RNA extraction, RNA purity test by Nanodrop Spectrophotometer, convert RNA become cDNA by Reverse Transcription–Polymerase Chain Reaction, DNA amplification using Polymerase Chain Reaction, electrophoresis, and visualize using UV transilluminator. The mosquitoes that had been collected identified as female *Ae. albopictus* based on their one thick white line on it's mesonotum without two curved line and their thread-like antennae, with short and rare hair. Visualization by UV transilluminator present that *Ae. albopictus* contain DENV-2 dengue virus. In 2013, DENV-3 appeared when DENV-1 become predominant during 2008-2013. It was predicted that DENV-3 would be an outbreak in next year. In fact, it turned out that DENV-3 was really became an outbreak in 2013-2016. It indicated the possibility that DENV-2 we have just discovered could become an outbreak in the future.

Key words: *Dengue virus serotype, Aedes, Endemic area, Surabaya-Indonesia, January 2020*

Introduction

Dengue Haemorrhagic Fever (DHF) or Haemorrhagic Dengue Fever is an infectious disease caused by the bite of the *Aedes* sp. which contains dengue virus. Dengue virus consists of 4 types of serotypes namely, DENV-1, DENV-2, DENV-3 and DENV-4. Number of DHF increase significantly, as recorded in 2015 there were 21,092 people and in 2016 an increase of 25,336 people (Rahayu and Ustiawan, 2013).

Ae. aegypti mosquito is the main vector causing

[1]Undergraduate Student

DHF while *Ae. albopictus* is a secondary vector causing DHF. Adult mosquitoes have the characteristics of a basic black colour with white spots on the body and legs. These white spots are actually scales that stick to the outside of the mosquito's body. On the dorsal chest (mesonotum) *Ae. aegypti* there is a picture of the left and right white curved line and in the middle there are 2 elongated white lines that resemble harp instruments (Rueda, 2004), while *Ae. albopictus* only has one white strip (Rahayu and Ustiawan, 2013).

In 2013, dengue disease has spread in 33 provinces in Indonesia. Also, in 2015 there were 1,071 people died from the total number of DHF sufferers that year amounted to 129,650 people. Incidence rate of DHF in Indonesia during 2015 was 50.57% and the Case Fatality Rate (CFR) was 0.83%. The number of cases in 2014 was 100,347 people with an IR of 39.80% and a CFR of 0.90% (Dinkesprov Jawa Timur, 2017).

One of methods that was used to detect dengue virus serotypes is Reverse Transcription - Polymerase Chain Reaction (RT-PCR) and Polymerase Chain Reaction (PCR). RT-PCR is a type of PCR, which is a technique for multiplying DNA sequences in large quantities and in a short amount of time through a process called Amplification. The working principle of RT-PCR is by transcribing or transforming RNA into complementary DNA (cDNA) using the help of the enzyme Reverse Transcriptase (Sudjadi, 2008).

This study aims to identify the occurrence of dengue virus in *Aedes* mosquito, due to the start of rainy season. Preliminary study would be needs as a mitigation for dengue outbreak, so that early detection could be a preventive way.

Materials and Methods

Material

Minimum Essential Medium (MEM) C6/36, Reverse transcriptase reagent (Invitrogen, USA), RNA extraction kit by QIAGEN Germany, dNTP, Forward Primer (D1), Reverse Primer (TS1, TS2, TS3, TS4), 5x FS Buffer, DTT, Super Script, nuclear free water, Promega master mix (USA), Agarose gel, 0.5X Buffer TAE, Ladder 100bp, Ethidium bromide, DENV-2 Surabaya strain with accesion number KT012509 as control positive, aspirator, Eppendorf tube, filter, microsentrifuge, vortex, Takara as PCR machine, electrophoresis machine, UV Transillumi-nator and Nanodrop spectrophotometer.

Identification of Mosquitoes

Adult mosquitoes were caught in the parking area at Institute Tropical Disease Universitas Airlangga using aspirator. Mosquito then freezed in -80 ^{o}C freezer. Female and male mosquitos were differentiated by looking their antennae and species confirmation can be done by observing lyra pattern on its head as mentioned in *Aedes* identification key (Departemen Kesehatan Republik Indonesia, 1989).

Preparation

Adult mosquito were suspended with 500 µL MEM C6/36 in the Eppendorf tube, then were pounded. Suspension was filtered and filtrate used for RNA extraction.

RNA extraction

Viral RNA was isolated by using Mini kit QIAGEN. Buffer AVL containing carrier RNA were pipetted 560 µL into 1.5 mL microsentrifuge tube. *Ae. Albopictus* were added to Buffer AVL-carrier RNA in the microsentrifuge tube. After mixing the compound by pulse-vortexing for 15 s, incubation at room temperature (15-25°C) were done. The tube was briefly centrifuged to remove drops from inside of the lid. Ethanol (96-100%) amount 560 µL was added to the sample to precipitate RNA and mixed by pulse-vortexing for 15 s. Then, the tube was briefly centrifuged. After that, 630 µL of the solution then carefully applied to the QIAamp Mini column and centrifuged at 6000 x g (8000 rpm) for 1 minute. The QIAamp Mini column then placed into a clean 2 mL collection tube. This step was repeated twice. After that, 500 µL of Buffer AW1 was added to the column and centrifuged at 6000 x g (8000 rpm) for 1 minute. QIAamp Mini column then placed in clean 2 mL collection tube and added 500 µL Buffer AW2. The column then centrifuged at full speed (20,000 x g; 14,000 rpm) for 3 minutes. The QIAamp Mini column was placed in a clean 1.5 mL tube and added 60 µL of Buffer AVE equilibrated to room temperature. After incubated at room temperature for 1 minute, the column was centrifuged at 6000 x g (8000 rpm) for 1 minute. RNA purity was tested using Spectrophotometer Nanodrop with abrsorbance 260/280.

Reverse Transcriptase - Polymerase Chain Reaction

RT-PCR (Reverse transcriptase-PCR) was applied to

convert target viral RNA to a DNA copy (cDNA) prior to enzymatic DNA amplification. Three target RNA was amplified in 14 µL volumes containing 1 µL dNTP and 0.5 µL Reverse Primer (TS1, TS2, TS3, TS4)], 7 µL Nuclear Free Water. The reactions were allowed to proceed in Takara (Japan) thermocycler programmed to incubate for 5 minutes at 65°C.

Next step, 4 µL 5x FS Buffer, 1 µL DTT and 0.5 µL Super Script enzyme. The compound then added to RT I tube amount 5.5 µL.

The reactions were allowed to proceed in Takara (Japan) thermocycler programmed to incubate for 1 h 5 s at 65 °C.

Amplification of Virus DNA

Polymerase Chain Reaction was done in 25µL volume consist of 12.5 µL Promega master mix, 2µL primer (1µL forward primer and 1µL reverse primer), 5.5 Promega nuclear free water and 5 µL sample. The reactions were allowed to proceed in Takara (Japan) thermocycler programmed to incubate for 3 h. Polymerase Chain Reaction was done in 3 stages: Denaturation (94 ^{o} C, 1 min), annealing (50 ^{o} C, 1 min), and Extension (72 ^{o}C, 2 min) in 35 cycle.

Sequence of Forward Primer (D1) and Reverse Primer (TS1, TS2, TS3, TS4) were shown in Table 1.

Agarose electrophoresis

To make electrophoresis gel, 1.5 gram Agarose were added into 100 mL 0.5X Buffer TAE. After Ladder and all samples were put in well, ran electrophoresis in 100 volt for 30 min. Staining for 30 min using Ethidium bromide was done to make RNA band so it could be seen more clearly. Gel that contained RNA was visualized in the UV Transilluminator.

Results and Discussion

Serotype identification needed to determine how many type of dengue viruses were circulated in a population (Sari *et al.,* 2012). More than one serotype in an area contributes to the high level of severity of dengue haemorrhagic fever (DHF) case. Second infection with different serotype cause worse effect to sufferers (World Health Organization, 2011).

Mosquitoes were collected from the parking area of Institute of Tropical Disease, Universitas Airlangga, because of the DHF vector mosquito preference for rather dark and humid place. Identification was carried out by stereo microscope, and all of three mosquitoes identified as *Ae. albopictus* based on the presence of a thick white line on their *mesonotum* (Rahayu and Ustiawan, 2013). The three mosquitoes identified as female based on their thread-like antennae, with short and rare hair (Levin, 2014) as shown in Figure 1. Female *Ae. albopictus* need blood for maturating her eggs. *Ae. albopictus* thick white line in Figure 2 and 3 were rather invisible due to their fragile scales have fallen off (Purnama, 2017).

Fig. 1. The first mosquito identified

MEM (Minimum Essential Medium) used in preparation process used for viral isolation. MEM allows dengue virus not to die when isolated. Besides MEM, PBS (Phosphat Buffered Saline) can also be used.

Table 1. Oligonucleotide primers used to amplify and type dengue viruses (Lanciotti *et al.,* 1992)

Primer	Sequence	Genome Position	Size (bp) of ampiflied DNA product (primer)
D1	5'-TCAATATGCT GAAACGCGCGAGAAACCG-3'	134-161	511
TS1	5'-CGTCTCAGTGATCCGGGGG-3'	568-586	482 (D1 and TS1)
TS2	5'-CGCCACAAGGGCCATGAACAG-3'	232-252	119 (D1 and TS2)
TS3	5'-TAACATCATCATGAGACAGAGC-3'	400-421	290 (D1 and TS3)
TS4	5'-CTCTGTTGTCTTAAACAAGAGA-3'	506-527	392 (D1 and TS4)

RNA extraction process requires buffer AVL to lyse viruses and mosquito cells. Carrier RNA contained in the buffer AVL is useful to increase the amount of isolated RNA. Mix by pulse-vortexing aims to yield homogenous solution. Incubation for 10 minute at room temperature provide an opportunity for buffer AVL to complete viral lysis and inactivate RNases. The addition of ethanol was to precipitate the viral RNA. Buffer AW1 and AW2 washed RNA from contaminating agent. RNA were eluted because of buffer AVE. as a result, filtrate contained total RNA.

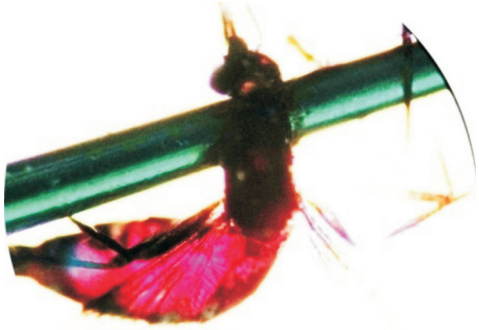

Fig. 2. The second mosquito identified

Fig. 3. The third mosquito identified

The result of RNA purity assay show that sample 1 had ratio of absorbance A260/A280 was 3.21, sample 2 was 3.32, and sample 3 was 3.19. According to Maharani *et al.* (2006), absorbance ratio (A260/A280) among 1.8-2.1 show the good quality of total RNA. Absorbance ratio less than 1.8 indicated that total RNA contaminated by protein (Imbeaud *et al.,* 2005), while absorbance ratio more than 2.1 indicated DNA contamination (Brisco *et al.,* 1997). The absorbance ratio value of all the samples were higher than 2.1, indicated that the samples were contaminated by DNA.

The solution from RNA extraction process contained total RNA which was contaminated by DNA, and had not been known whether it contained dengue viral RNA or not. Therefore, RT-PCR and PCR were required to do. RT-PCR was done to convert total RNA became cDNA. By using reverse primer (Table 1). Furthermore, the cDNA samples were amplified by PCR, which consists of 3 steps. Denaturation step took 1 minute at 94 °C, annealing took 1 minute at 50°C, and extension took 2 minute at 72 °C. Electrophoresis were performed after PCR to visualize the dengue viral RNA, if any. The result of electrophoresis visualized with UV transilluminator, can be seen in Figure 4. The DNA band of positive control, which contains dengue viral DNA, appear on 119 bp. The DNA band of all three samples also appear at 119 bp. Therefore, it indicated that all of the samples contained dengue viral DNA. According to Lanciotti *et al.* (1992), DNA band which was appeared on 119 bp is DNA band of type DENV-2 dengue virus.

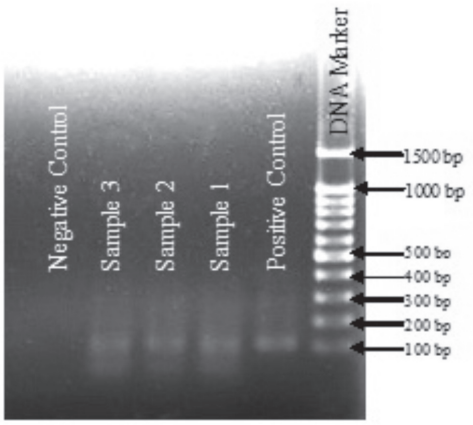

Fig. 4. Visualization with UV transilluminator

The research from Kotaki *et al.* (2014) shows that DENV-1 was the predominant type in Surabaya at 2008-2013, meanwhile on January 2013 DENV-3 appeared for the first time. In the next research, Kotaki *et al.* (2014) found that 2 of 3 patients from Surya Maternal and Child Health Hospital were infected by DENV-3 and predicted it was potentially cause endemic outbreak in Surabaya for the next years. The prediction was proved in 2019. Utama *et. al.* (2019) found that in 2013-2016 DENV-3 became predominant among other serotype. The appear of dengue virus serotype is like a cycle, where another strain which was not a predominant had potential to become an outbreak in the next time. As the dis-

covery of DENV-2 in this journal, it is possible that this serotype will become an outbreak in the future. Further studies on the possibility of outbreaks of other dengue virus serotype, especially DENV-2 was needed. The effort of health officer to monitor and prepare for eradication of the outbreak will be very necessary.

In conclusion, *Ae. albopictus* that had been found in Institute of Tropical Disease's parking area contain DENV-2 serotype dengue viral. This serotype has potential to become an outbreak in the future. Further studies on the possibility of outbreaks of other dengue virus serotype, especially DENV-2 was needed.

Acknowledgement

Thank you to the Center of Excellence (COE) program by the Ministry of Research, Technology, and Higher Education of the Republic of Indonesia. This study was funded by Program Hibah Riset Mandat Universitas Airlangga (HRMUA) grant from Universitas Airlangga.

References

Brisco, P., Sankbeil, J. and Kephart, D. 1997. RNA Purification: A Rapid and Versatile Protocol for The Isolation of Total RNA. *Promega Notes*. 64: 07

Departemen Kesehatan Republik Indonesia. 1989. *Kunci Identifikasi Aedes Jentik dan Dewasa di Jawa*. Ditjen P2M Dan PLP. Departemen Kesehatan RI, Jakarta.

Dinkesprov Jawa Timur. 2017. *Profil kesehatan Provinsi Jawa Timur tahun 2016*. Dinas Kesehatan Provinsi Jawa Timur. Kota Surabaya. Available: https://www.depkes.go.id/resources/download/profil/PROFIL_KES_PROVINSI_2016/15_Jatim_2016.pdf

Imbeaud, S., Graudens, E., Boulanger, V., Barlet, X., Zaborski, P., Eveno, E., Mueller, O., Schroeder, A. and Auffray, C. 2005. Towards standization of RNA quality assessment using user-independent classifiers of microcapillary electrophoresis traces. *Nucleic Acids Research*. 33(6) : 1-12.

Kemenkes, RI. 2016. *Profil kesehatan Indonesia tahun 2015. Kementerian Kesehatan RI*. Jakarta. Available: https://www.depkes.go.id/article/view/16091600001/-profil-kesehatan-indonesia-tahun-2015.html

Kotaki, T., Yamanaka, A., Mulyatno, K.C., Churrotin, S., Labiqah, A., Sucipto, T.H., Soegijanto, S., Kameoka, M. and Konishi, E. 2014. Continuous Dengue Type 1 Virus Genotype Shifts Followed by Co-Circulation, Clade Shift and Subsequent Disappearance in Surabaya, Indonesia, 2008-2013. *Infection, Genetics and Evolution*. 28 : 48-54.

Kotaki, T., Yamanaka, A., Mulyatno, K.C., Labiqah, A., Sucipto, T.H., Churrotin, S., Soegijanto S., Konishi, E. and Kameoka, M. 2014. Phylogenetic Analysis of Dengue Virus Type 3 Strains Primarily Isolated in 2013 from Surabaya, Indonesia. *Japanese Journal of Infectious Diseases*. 67(3): 227-229.

Lanciotti, R.S., Calisher, C.H., Gubler, D.J., Chang, G.J. and Vorndam, A.V. 1992. Rapid Detection and Typing of Dengue Viruses from Clinical Samples by Using Reverse Transcriptase-Polymerase Chain Reaction. *Journal of Clinical Microbiology*. 30(3): 545-551.

Levin, M.L. 2014. Medical Entomology for Student 5th Edition. *Emerging Infectious Diseases*. 20(8): 1428

Maharani, A., Sucipto, T.H., Setyawati, H., Churrotin, S., Amarullah, I.H., Wardhani, P., Aryati, Ueda, S. and Soegijanto, S. 2018. RNA isolation of dengue virus type 1 with different precipitation solvents: dimethyl sulfoxide, acetone, and ethanol 70%. *Indonesian Journal of Tropical and Infectious Disease*. 7(3): 62-66

Purnama, S.G. 2017. Diktat Pengendalian Vektor. Program Studi Ilmu Kesehatan Masyarakat Fakultas Kedokteran Universitas Udayana. Available: https://simdos.unud.ac.id/uploads/file_pendidikan_dir/22d82a3dbab6e380e1 aaf347e86dc055.pdf

Rahayu, D.F. and Ustiawan, A. 2013. Identifikasi *Aedes aegypti* dan *Aedes albopictus*. *Jurnal Litbang Pengendalian Penyakit Bersumber Binatang Banjarnegara*. 9(1): 7-10.

Rueda, L.M. 2004. Pictorial Keys for the Identification of Mosquito (Diptera:Cullicidae) Associated with Dengue Virus Transmission. *Zootaxa*. 589: 1-60

Sari, T.F., Joharina, A.S. and Anggraeni, Y.M. 2012. *Identifikasi Serotype Virus Dengue pada Nyamuk Aedes aegypti dan Aedes albopictus di Kota Salatiga dengan Metode RT-PCR*. Salatiga: Balai Besar Penelitian dan PengembanganVektor dan Reservoir Penyakit. Available: http://repository.litbang.kemkes.go.id/575/

Sudjadi, 2008. *Bioteknologi Kesehatan*. Edisi 1. Kanisius, Yogyakarta.

Utama, I.M.S., Lukman, N., Sukmawati, D.D., Alisjahbana, B., Alam, A., Murniati, D., Utama, I.M.G.D.L., Puspitasari, D., Kosasih, H., Laksono, I., Karyana, M., Karyanti, M.R., Hapsari, M.M.D.E.A.H., Meutia, N., Liang, C.J., Wulan, W.N., Lau, C., Parwati, K.T.M. 2019. Dengue viral infection in Indonesia: epidemology, diagnostic challenges, and mutations from an observational cohort study. *PLOS Neglected Tropical Diseases*. 13(10): 1-19

World Health Organization, Regional Office for South-East Asia. (ý2011)ý. Comprehensive Guideline for Prevention and Control of Dengue and Dengue Haemorrhagic Fever. Revised and expanded edition. WHO Regional Office for South-East Asia. Available: https://apps.who.int/iris/handle/10665/204894