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Research Report

RNA ISOLATION OF DENGUE VIRUS TYPE 1 WITH DIFFERENT PRECIPITATION SOLVENTS: DIMETHYL SULFOXIDE, ACETONE, AND ETHANOL 70%

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ABSTRACT

Dengue Hemorrhagic Fever (DHF) is caused by dengue viruses that belong to Flaviviridae. The disease is known to be caused by 4 types of dengue viruses, namely DENV-1, DENV-2, DENV-3, and DENV-4 associated with antigenic. Dengue virus is a virus RNA that causes illness with clinical manifestations of Dengue Fever, Dengue Hemorrhagic Fever and Dengue Shock Syndrome. The aim of research was to determine the effectiveness of dimethyl sulfoxide, acetone, and ethanol 70% as precipitation solvent in the process of RNA isolation. The method used was Reverse Transcription - Polymerase Chain Reaction (RT-PCR) and Polymerase Chain Reaction (PCR) with specific primers for dengue virus type 1 (DENV-1). RNA isolation can be done easily using an RNA Isolation Kit. Use of RNA Isolation Kit results in a purer RNA isolate from contaminants and from RNA degradation. In generally the isolation is using cold ethanol / alcohol with concentration 90-95%. Ethanol / Alcohol does not dissolve RNA and light density of alcohol lighter than water makes RNA rise and hover on the surface. In RNA isolation solvent precipitation that used are acetone, ethanol 70%, and DMSO. In qualitative RNA measurements using agarose gel electrophoresis and was examined under the UV light-illuminator and quantitative RNA measurements using Nanodrop spectrophotometry with absorbance ratio at 260/280 and 260/230 showed a good result indicated by the appearance of the band on electrophoresis results in PCR. While the measurement quantitatively is showed that there was still protein contamination but the results are quite good because it does not much different from the ratio set in the reference. Acetone, ethanol 70%, and DMSO can be used as a substitute of 96% ethanol in the process of RNA isolation in DENV-1 virus and can also be applied to other dengue virus because the structure of the 4th antigen serotype is very similar one with the other and no effect.

Key words: *RNA, Precipitation Solvents, DMSO, Acetone, Ethanol*

ABSTRAK

Demam Berdarah Dengue (DBD) disebabkan oleh virus dengue milik Flaviviridae. Penyakit ini diketahui disebabkan oleh 4 jenis virus dengue, yaitu DENV-1, DENV-2, DENV-3, dan DENV-4 yang terkait dengan antigenik. Virus Dengue adalah virus RNA yang menyebabkan penyakit dengan manifestasi klinis Demam Berdarah Dengue, Demam Berdarah Dengue dan Syok Dengue Shock. Metode yang digunakan Reverse Transcription - Polymerase Chain Reaction (RT-PCR) dan Polymerase Chain Reaction (PCR) dengan primer spesifik untuk virus dengue tipe 1 (DENV-1). Isolasi RNA dapat dilakukan dengan mudah menggunakan RNA Isolation Kit. Penggunaan RNA Isolation Kit menghasilkan isolat RNA yang lebih murni dari kontaminan dan dari degradasi RNA. Pada umumnya isolasi menggunakan etanol/ alkohol dingin dengan konsentrasi 90-95%. Etanol/Alkohol tidak melarutkan RNA dan kerapatan ringan alkohol lebih ringan daripada air membuat RNA naik dan melayang di permukaan. Pada pengendapan pelarut RNA terisolasi yang digunakan adalah aseton, etanol 70%, dan DMSO. Pada pengukuran RNA kualitatif menggunakan elektroforesis gel agarose dan diperiksa di bawah sinar-iluminator UV dan pengukuran RNA kuantitatif menggunakan spektrofotometri Nanodrop dengan rasio absorbansi pada 260/280 dan 260/230 menunjukkan hasil yang baik yang ditunjukkan oleh kemunculan band hasil elektroforesis pada PCR. Sedangkan pengukuran secara kuantitatif menunjukkan bahwa masih ada sedikit kontaminasi protein namun hasil tersebut cukup

baik karena tidak berbeda jauh dari rasio yang ditetapkan dalam referensi. Sehingga dapat disimpulkan hasil tersebut menunjukkan bahwa aseton, etanol 70%, dan DMSO dapat digunakan sebagai pengganti etanol 96% dalam proses isolasi RNA pada virus DENV-1 serta dapat juga diaplikasikan pada virus dengue yang lain karena struktur antigen ke-4 serotipe ini sangat mirip satu dengan yang lain dan tidak berpengaruh.

Kata kunci: *RNA, Presipitasi Pelarut, DMSO, Aseton, Etanol*

INTRODUCTION

Dengue is appeared in two forms, classic dengue fever and severe form. In severe form, Dengue Hemorrhagic Fever (DHF) and Dengue Shock Syndrome (DSS) can caused abdominal bleeding, hemorrhage and circulatory failure. If this severe form is not treated with prompt this will lead to the fatal cases.[1] Dengue Hemorrhagic Fever (DHF) caused by dengue viruses is still a major problem in tropical countries.[2] Dengue Hemorrhagic Fever (DHF) are the most rapidly spreading vector-borne diseases with approximately 50 million cases of infection worldwide.[3] In the last decades, dengue has emerged as a prime health concern in tropical and subtropical expanse of the world resulting in increased mortality every year.[4] Indonesia is the one of the many tropical countries that are affected by the dengue virus (DENV). The main clinical manifestations, namely Dengue Hemorrhagic Fever (DHF) and dengue shock syndrome (DSS), are responsible for high morbidity and mortality rates every year. Over 40% (2.5 billion) of the population in 100 tropical and subtropical countries continue to live under the threat of contracting dengue infection. It is estimated that 100 million cases of DF, 500,000 cases of DHF, and 25,000 deaths are reported annually worldwide.[5] Increases in human population size, dengue vector-density and human mobility cause rapid spread of dengue virus in Indonesia. Dengue virus is transmitted through the bite of a female mosquito. Transmission can occur when Aedes female mosquitoes are sucking blood of people infected with dengue virus and the mosquito will soon bite others as well.

The process of isolating RNA was through several stages and in that stage, the role of solvent is very important that is to know the level of effectiveness of the solvent used and its impact on the results of the isolation. In a previous research, RNA isolation protocol were based on Cetyltrimethylammonium Bromide (CTAB).[6,7,8,9] and two successive precipitations with 10 M lithium chloride (LiCl).[10,11,12] The good parameters in CTAB depend on several factors. First, the NaCl concentration must be above 1.0 M to prevent the formation of CTAB-RNA complexes. Then, extracts and CTAB-containing cell solutions should be stored at room temperature since the CTAB-DNA complex is insoluble in temperatures below 15°C. And the last, the use of CTAB with good purity will determine the purity of RNA obtained and with very little polysaccharide contamination, with CTAB method will also be obtained RNA with a thin band located far below the DNA band. The existence of the RNA band depends on the extracted material.[13] Although LiCl is commonly used to precipitate RNA, but this method has received little attention and not effective with low concentration. Generally we are used ethanol or isopropanol in the precipitation stage. Both of these compounds will precipitate RNA in the aqueous phase so that the RNA agglomerates to form a fiber structure and pellets are formed after centrifugation. In this research we used several kinds of solvents that can be an alternative to a solvent commonly used in RNA isolation, the topic we are raising is RNA Isolation of Dengue Virus Type 1 with Different Precipitation Solvents: dimethyl sulfoxide (DMSO), acetone, and ethanol 70%. Dimethyl sulfoxide (DMSO), acetone, and ethanol 70%which has lower constant dielectric compared to water increases the interaction of the salt and the Coulomb force of attraction between the cation and the negatively charged nucleic acid backbone (that is, the resistance from the solvent's electric field sufficiently diminishes to gain efficient interaction; the solvation shells surrounding the solute's charges depletes).[14]

Table 1. Oligonucleotide primers used to amplify and type dengue viruses.[15,16,17]

Primer	Sequence	Genome position	Size in bp, of amplified DNA product (primers)
D1	5'-TCAATATGCTGAAACGCGCGAGAAACCG-3'	134-161	511
D2	5'-TTGCACCAACAGTCAATGTCTTCAGGTTC-3'	616-644	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)

MATERIAL AND METHOD

Dengue virus type 1 (DENV-1) was isolated from Surabaya (Genbank accession AB915377), Acetone (Merck, Germany), Dimethyl Sulfoxide (DMSO) (Merck, Germany), Ethanol 96% (Merck, Germany), RNA isolation kit (QIAamp Mini Kit) was supplied by Qiagen from USA, master mix PCR was supplied by Promega from USA, reagent for RT-PCR was supplied by Invitrogen from Germany, nuclease free water (NFW) was supplied Qiagen from USA, Ethidium bromide (Merck, Germany), TAE buffer (Promega, USA), Agarose (Promega, USA), and primer are demonstrated in the Table 1.

RNA Isolation

For RNA, isolation from Vero infected cells has used concentration from the reagent. Samples were taken from refrigerator and were melted. Sample were mixed 2.24 ml AVL and 22.4 µl AVE, this composition was already included in the protocol of the kit that has been purchased. Mixing this solution with vortex for 15 s. After centrifugation, the resulting RNA pellet was washed with ethanol 560 µl. Then, move 630 µl to mini column QiAamp. The mixture was centrifuged at 8,000 x g for 1 min, and the water was removed and combined with an equal volume of solvent to precipitate the RNA. After that, add 500 µl buffer AW 1, centrifuged at 8,000 x g for 1 min. The next step is add 500 µl buffer AW 2, centrifuged at 14,000 x g for 3 min then remove the remaining water and move to the new tube and then centrifuged again at 14,000 x g for 3 min and after finishing put it down to the eppendorf tube. Add with AVE Buffer 40 µl then centrifuged at 8,000 x g for 1 min. For the last, keep it in temperature -80°C. In this research we change precipitation solvents with DMSO, Acetone, and Ethanol 70%. RNA measurements were quantitatively performed using nanodrop spectrophotometry with an polysaccharides absorb most UV light at λ230 nm and protein at λ280 nm.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

RT-PCR process has used concentration from the reagent. Mix dNTP, primer, NFW, and RNA then was centrifuged for 1 min. Then, the mixture was put in Thermocycle with temperature 65°C for 5 min. For the next step, master mix that consist of FS Buffer, RNAse Out, DTT and superscript was made. The mixture was centrifuged at 8,000 x g for 1 min. The first master mix from Thermocycle was mixed with the second master mix, then it was centrifuged for 1 min and was put in to Thermocycle at 50°C for 60 min, and then the process was continued for temperature 85°C for 5 min.

Isolation of DNA by PCR

12.5 µL master mix was mixed with 5.5 µL nuclease free water (NFW), 2 µL primer, and 5µL cDNA from RT-PCR result. Then, was put in to micro tube. The mixture was centrifuged at 8,000 x g for 1 min. After that the solution was inserted into the Thermocycle.

Agarose gel electrophoresis

Some of the electrophoresis process steps mixing 1 µL marker, 6 µL TAE buffer, and 3 µL DNA. The gel was run for 30 min at 100 volt and was stained with ethidium bromide. The bands were visualized on an ultraviolet trans-illuminator.

RESULT AND DISCUSSION

In this report, we have described solvent that we have used for isolation of RNA. In this research, we have been able to complete the RT-PCR assay, starting from RNA isolation and completing with agarose gel analysis. In addition, dengue virus was consists of four serotypes (DENV-1, DENV-2, DENV-3 and DENV-4).[18,19,20] In Indonesia there were dominance serotype DENV-2, followed DENV-3 in 2003 to 2005.[21] Virus serotype can be demonstrated by molecular techniques such as PCR and RT-PCR. Isolation of RNA can be done through several stages as described in the research method. The result was obtained from the isolation process were used for the RT-PCR process. Figure 1 was showed a serum samples that were obtained from Surabaya. The results were obtained throughout the sample is positive, there is no dengue virus DNA bands appear, actually the bands were visible but not intensive.

Isolation of RNA with PCR was done in a similar way to RT-PCR but with different temperature variations. RT-PCR was used temperature 45ºC whereas PCR used temperature 65ºC. The data which was obtained from isolation DNA by PCR was showed the good results. When ethanol was removed and the pellet was drained in a tube, the remaining pellets in the tube were concentrated RNA. The process of re-precipitation with ethanol before pellet was allowed to increase the degree of purity of isolated RNA.[22] After the isolation process, the obtained DNA can be concentrated by precipitation. Commonly was used ethanol or isopropanol in the precipitation stage. Both of these compounds will precipitate RNA in the aqueous phase so that the RNA agglomerates to form a fiber structure and pellets were formed after centrifugation. At this precipitation stage, the precipitated RNA will be separated from the remaining DNA and protein residues. The residue also undergoes coagulation but there was no fiber structure and was in the form of granular precipitates.[23] When solvents was removed and the pellet was dried in a tube, the remaining pellets in the tube were concentrated RNA. The process of precipitation with solvents before the pellet was dried can increase the degree of purity of isolated RNA. In the isolation process, the principle is to use organic solvents, carry out with a substance that is insoluble in water, dissolve in an organic solvent, mix with water, then add distilled water under certain conditions.[24] Ethanol was a versatile solvent, water soluble and other organic solvents. So, ethanol was the most important solvent option used in the RNA isolation process. Acetone, ethanol 70%,and DMSO precipitation were efficient method for isolation

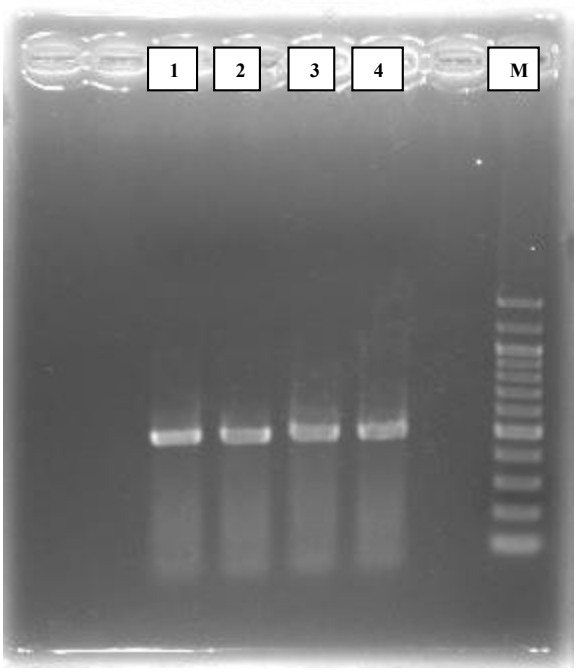

Figure 1. Results of electrophoresis of PCR with solvent (1) DMSO, (2) Ethanol 70%, (3) Acetone, (4) Ethanol 96%, and (M) Marker.

of RNA. They are including in polar solvents and have almost the same solubility properties as ethanol. Some important properties of solvents include ability to solubility, the velocity evaporates, boiling route, specific gravity, flashpoint. This equation was allowed them to be used as a replacement solvent when ethanol was absent. The appearance of the ribbon was clearly showed that acetone, ethanol 70%, and DMSO can be used instead of ethanol. In addition, these solvents were easier to obtain than other organic solvents. The results were positive, they were showed dengue virus DNA bands. Positive control was used the dengue virus serotype DENV-1 (482bp).

The next step is to know RNA measurements were quantitatively performed using nanodrop spectrophotometry with an absorbance ratio of 260/280 and 260/230. Absorbance data was showed in Table 2. The principle of nanodrop spectrophotometric work is pure RNA which capable of absorbing ultraviolet light due to the presence of purine and pyrimidine bases.[25]

The presence of contaminants can also be known through spectrophotometer. Polysaccharides were absorbed most UV light at λ230 nm and protein at λ280 nm. The level of purity of RNA can be known by measuring the amount of sample absorbance at λ230 nm, λ260 nm, and λ280 nm, then measuring large comparison (ratio) A260 to A280 and ratio A260 against A230.[26] Pure RNA isolate has an A260/A280 ratio of 2.0±0.1. A low A260/A280 ratio indicates protein contamination. From the test results can be known that at A260/280 nm much samples have a ratio of more than 2.0. It can be stated that the RNA isolate

Table 2. The data result of NanoDrop Spectrophotometry for RNA samples

Sample	A260/280 nm	A260/A230 nm
DENV1 – Ethanol 70%	3.28	0.38
DENV1 – Ethanol 96%	3.25	0.28
DENV1 – Acetone	3.16	0.39
DENV1 – DMSO	3.20	0.48

is contaminated with proteins even it is not too much. In the above data can be seen nanodrop results for acetone on A260/280 nm lower than the other three solvents. In addition, if sorted from the yield of nanodrop, ethanol 70% has the highest value compared with the other three solvents so that the value of protein contamination can be said more than 96% ethanol, acetone, or DMSO. The test results on A260/A230 was showed that no sample less than 1.5 which means the results of RNA still contain other contaminants, pure RNA isolate has an A260/A230 ratio of 2.0-2.4.[27] So it can be concluded that 96% ethanol, acetone, and DMSO can be used as precipitation solvents in RNA isolation because the protein concentrations of nanodrop yields show good results. The qualitative results indicate that the three solvents can be used as precipitation of solvent in the process of RNA isolation, which were indicated by the appearance of the band on the electrophoresis results of PCR. The results were showed that acetone, ethanol 70%, and DMSO can be used as a substitute of 96% ethanol in the process of RNA isolation in DENV-1 virus and can also be applied to other dengue virus because the structure of the 4th antigen serotype is very similar one with the other and no effect.

Based on previous research on the epidemiology of dengue fever in Indonesia, WHO South-East Asia Regional Office (SEARO), Indonesia did not report laboratory-confirmed data in period 2000-2013. Also no active data were found for the study period, and very few dengue case were reported on the SEARO website, although dengue is considered to be endemic countries. Another limitation is that we did not further elaborate on circulation of the predominant serotype, as only qualitative data were available from passive surveillance data.[28] In Surabaya-Indonesia, 2012, DENV-1 was reported dominant serotype with 90.7%, followed by DENV-2 (9.3%), for DENV-3 and DENV-4 were not detected. In 2013, DENV-1 was reported dominant serotype also with 66.7%, DENV-2 (27.5%), DENV-3 (5.8%), and DENV-4 were not detected.[18] In 2014, DENV-2 was reported dominant serotype with 92.8%, DENV-1 (7.2%), for DENV-3 and DENV-4 were not detected. The same time, Madura island isolates was showed high nucleotide similarity to other Indonesia isolates, indicating frequent virus circulation in Indonesia.[29] The results of the present study highlight the importance of help viral isolation in dengue endemic areas to obtain a cleare understanding of the dynamics of DENV in Indonesia.

CONCLUSION

In this study, RNA measurements were performed qualitatively using agarose gel electrophoresis and examined under a UV trans-illuminator. The quantitative RNA measurement was used nanodrop spectrophotometry with absorbance ratio at 260/280 and 260/230. RNA measurements were quantitatively performed using nanodrop spectrophotometry with an absorbance ratio of 260/280 and 260/230 was showed good results, ethanol 70% has the highest value compared to the other three solvents so that the value of protein contamination can be said more than 96% ethanol, acetone, or DMSO.While the results on absorbance 260/230 was showed that no sample less than 1.5 which means the results of RNA still contain other contaminants, pure RNA isolate has an A260 / A230 ratio of 2.0-2.4. The qualitative measurements were showed that in PCR, the appearance of the ribbon was signified that acetone, ethanol 70%, and DMSO can be used instead of ethanol, so if ethanol 96% is not available in the laboratory, we can be used DMSO, acetone, and ethanol 70% as precipitation of solvent in the process of RNA isolation in DENV-1 virus and they can also be applied to other dengue virus because the structure of the 4th antigen serotype is very similar with the other and there was.

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