BioEksakta: Jurnal Ilmiah Biologi Unsoed
Volume 2, Nomor 1 (2020): 56 - 61
E-ISSN: 2714-8564

Reverse Transcriptase PCR (RT-PCR) for Detection of Dengue and Chikungunya Virus of Mosquito *Aedes aegypti* In Sokaraja

Dyah Retno Annisa*, Endang Srimurni Kusmintarsih, Trisnowati Budi Ambarningrum

Fakultas Biologi, Universitas Jenderal Soedirman
Jalan dr Suparno 63 Purwokerto 53122
*email: dyahannisa@gmail.com

Article History:

Received : 29/08/2019
Accepted : 03/03/2020

Abstract
Aedes aegypti is a primary vector of dengue and chikungunya virus. Dengue virus causes a disease called dengue fever (DF). Meanwhile, Chikungunya virus causes Chikungunya fever (CF). These diseases involve three organisms, namely virus, mosquito *Aedes* sp., and human. The transmission of dengue and chikungunya virus is related to the population of *Ae.aegypti.* Banyumas regency is one of the regions with many cases of dengue and Chikungnya virus infections, particularly in Purwokerto, Sokaraja, and Cilongok sub-district. Up to this time, there is no medicine and vaccine provided to treat these viruses effectively. Thus, detection of the virus inside vector will be effectively performed in order to predict the transmission risk of dengue and chikungunya virus. This research aimed to know the molecular detection of dengue and chikungunya virus on adult *Ae.aegypti* mosquito in Sokaraja Region, Banyumas Regency. The survey was done by a cross-sectional method in Sokaraja sub-district from May 2019 – March 2019. Furthermore, technical sampling used was a purposive sampling method of adult *Ae.aegypti* using BG-Sentital Trap, followed by molecular detection of dengue virus using Two-step RT-PCR and chikungunya gene virus using RT-PCR. Molecular detection of DENV and CHIKV of mosquitoes which collected from Sokaraja region showed a negative result means that Karangrau, Karangnanas, Kalikidang, and Sokaraja Kulon are free from Dengue and Chikungunya virus
Keywords*:* *Aedes aegypti, Sokaraja, dengue, chikungunya, RT-PCR*

INTRODUCTION

Aedes aegypti is mosquito originated from Africa whose population is influenced by climatic factors such as the amount of rainfall, temperature, and humidity (Kraemer et al., 2015; Nasir et al., 2017). Unstable climates and high intensity of rainfall increase the potential of *Ae.aegypti* larvae to develop rapidly (Banyumas Regency Health Department, 2015). According to Sunaryo & Pramestuti (2014), larvae of *Ae.aegypti* develop rapidly at the beginning and the end of rainy season. It happens because usually in this time, rain occurs not frequently causes eggs of the mosquito to develop into larvae and further stages. High-density larvae of *Ae. aegypti* also caused by the lack of attention of people around towards environmental hygiene, especially water containers. *Ae.aegypti* mosquito is responsible for transmitting arboviruses, including dengue virus and chikungunya virus (Muktar et al., 2016).

Dengue virus (DENV) causes a systemic viral disease called dengue fever, which transmitted throughout tropical and sub-tropical regions (Wahyono et al., 2017). World Health Organization (WHO) estimated that 50-100 million infections of dengue virus occur per year in more than 100 endemic countries with 20000 deaths and Asia Pacific is the most region exposed to dengue virus (WHO, 2012).

Indonesia is one of the endemic countries with many cases in dengue infection since 1968 when the first case of dengue is reported from Jakarta and Surabaya (Karyanti et al., 2014). There were 58 people infected and 24 people died. Distribution of dengue virus is related to the increasing mobility and density of the population (Ministry of Health Republic of Indonesia, 2010). In 2015, there were 126.675 cases of Dengue Hemorrhagic Fever (DHF) in 34 provinces of Indonesia, and 1.229 cases died. Those amounts are higher compared to the cases in 2014 with 100.347 cases DHF, and 907 people died (Ministry of Health Republic of Indonesia, 2016).

In Central Java province, there are 35 regencies have been contracted by dengue fever disease. Banyumas Regency is part of Central Java became the endemic area of Dengue virus with Infection Rates (IR) reaches 11,05 per 100.000 of the population (Central Java Health Office, 2017). The high IR caused by the failure of elimination activities of mosquito breeding places (Banyumas Regency Health Department, 2015). In 2014, Karangrau, Sokaraja Kulon, and Wiradadi in Sokaraja, parts of Banyumas Regency, are included in the endemic area of the Dengue virus (Sus, 2015).

Chikungunya virus (CHIKV) causes a fever followed by rash and severe joint pain called chikungunya fever (Zeller et al., 2016). In Indonesia, the first case of Chikungunya was reported in 1973 in Samarinda. While, in Central Java, the first case was reported in 2002 in Klaten Regency. Chikungunya case is increased during 2005 – 2006 from 46 cases become 86 cases, and in 2007 increased to 2801 cases (Susanti et al., 2014). Besides Klaten Regency, other regions with many cases of chikungunya fever found in Banyumas, Semarang, Banjarnegara, and Purbalingga (Pratama & Pawenang, 2017). In 2009 chikungunya virus infected 50 up to 70 people average in 20 villages in Cilongok region, part of Banyumas regency (Mut, 2009). While in 2013, 25 people were infected by chikungunya virus in Purwokerto (Tut, 2013). In the same year, 20 people in Sokaraja Lor village were infected by Chikungunya virus (Harian Banyumas, 2013). Based on many cases of dengue and chikungunya virus in Sokaraja sub-district, therefore, this research was done in this area especially villages in Sokaraja namely Karangrau, Karangnanas, Kalikidang, and Sokaraja Kulon.

Although scientist has developed a vaccine for preventing Chikungunya disease (Mahmud et al., 2017), there is no licensed vaccine against Dengue. Based on this situation, it is necessary to do direct detection of dengue and chikungunya virus using reverse transcription-polymerase chain reaction (RT-PCR) (Farraudière et al., 2017).

According to the above description, the distribution of Dengue and Chikungunya virus that infects adult *Ae.aegypti* mosquito in Sokaraja are still not known. The objectives of the research are to know the detection of dengue and chikungunya virus on adult *Ae.aegypti* mosquito in Sokaraja Region, Banyumas Regency. Hopefully, the research will be useful as information to the government and public about the preventive and elimination activities of *Ae.aegypti* to control the transmission of Dengue and Chikungunya virus in Sokaraja.

MATERIAL AND METHOD

The tools used in this research were BG sentinel trap, Erlenmeyer, tray, camera, collection bottle, microtube 0.2 ml and 1.5ml, microcentrifuge, spinner, grinder, vortex, freezer, water bath, micropipette and tip, incubator, PCR machine, microtube rack, microwave, electrophoresis set, and UV transilluminator.

The materials used in this research were adult mosquitos of *Aedes aegypti*, label, 70% alcohol, RNA isolation from mosquito, Dengue virus, Chikungunya virus, Purelink™ Viral RNA/DNA Mini Kit (Invitrogen- Life Technologies, USA), Superscript™ III First-Strand Synthesis System for RT PCR (Invitrogen, CA, USA), My Taq™ HS Red Mix (Bioline, Taunton, MA, USA), agarose 1.5 %, TAE buffer, DNA ladder 100bp, loading buffer, SYBR Green, Universal Dengue virus primer (D1 and D2) 511 bp and specific serotype Dengue virus primer are (TSl 482 bp, TS2 119 bp, TS3 290 bp, and TS4 392 bp) from Integrated DNA Technologies (IDT, USA), Chikungunya virus primer (CHIK-1 and CHIK-2) 330 bp from (Macrogen, Seoul, South Korea), and tissue.

A sampling of adult *Ae. aegypti* was conducted at the houses of residents in Sokaraja region, Banyumas regency. Dengue and Chikungunya virus was detected in Molecular Biology Laboratory, Faculty of Biology and Research Laboratory, Jenderal Soedirman University. The research was conducted in May 2019 – July 2019.

The research design used in this research was a survey method with cross-sectional approaches. The Cross-sectional survey was conducted by collecting different data at one point in time. Locations of the survey were in the Sokaraja region (Karangrau, Karangnanas, Kalikidang, and Sokaraja Kulon). The sampling technique used in this research was purposive sampling. The parameter used was gene band appearance visualized using a UV transilluminator.

Molecular Detection of CHIKV and DENV from Mosquito

1) RNA Extraction

The DNA Extraction process of DENV and CHIKV was performed using Purelink™ Viral RNA/DNA Mini Kit (Invitrogen- Life Technologies, USA) Cat. 12280050. This procedure is according to Sari, *et al* (2012) with modification, adult *Ae.aegypti* were put into microtube *Eppendorf* 1.5 ml, then 200 µl DEPC water was added. After that, mosquitoes were ground using grinders,

Mosquito lysate as much as 200 µl were added by 20 µl proteinase-K and 200 µl lysis buffer (containing 5,6 µg carrier RNA), then homogenized using vortex for 15 minutes. The sample was incubated for 15 minutes at 56°C, then spin at low speed to remove any drop inside of the lid. After that, the lysate was added by 250 µl ethanol absolute then it was homogenized using vortex for 15 minutes. After that, it was incubated at room temperature (RT) for 5 minutes, followed by a centrifugation process to remove any drop inside of the lid. The mosquito lysate removed into a viral spin column which paired with a collection tube. Then, it was centrifuged for one minute at 6800x g, followed by incubation at room temperature (25ºC). The collection tube was moved then the viral spin column was arranged into the washing tube. Wash Buffer 500 µl was added, it was centrifuged for one minute at 6800x g, followed by incubation process at RT (25ºC) then filtrate and the washing tube removed. That viral spin column was arranged into the new washing tube. Washing Buffer 500 µI was

added, it was centrifuged for one minute at 6800x g, followed by incubation process at RT (25ºC) then filtrate and the washing tube are removed. That viral spin column was arranged into the new washing tube. After that, it was centrifuged at 13000x g for one minute, followed by incubation process at RT (25ºC) then filtrate and washing tube are removed. The viral spin column was arranged into the new recovery tube, RNAse free water 50 µI was added followed by incubation process at RT (2 ºC) for one minute. This step was repeated two times. After that, it was centrifuged at 13000x g for a minute, followed by the incubation process at RT (25ºC). The viral spin column then removed; RNA viral was stored at -80ºC.

2) RT-PCR Amplification of CHIKV RNA and DENV RNA (First Step)

According to Organji, *et al.* (2017) with modification, RNA virus was converted to cDNA by Superscript™ III First-Strand Synthesis System for RT PCR (Invitrogen, CA, USA) Cat.No.18080-051. The process was starting by mixing reagent for cDNA that was made in RNAse- free microtube 0,2 ml with the total per reaction is 10 µl contained 7 µl total RNA sample, 1 µl 50 µM oligo$(dT)_{20}$, 1 µl dNTP mix (10 mM), 1 µl DEPC water. Mix reaction was incubated at 65°C for 5 minutes, then placed on ice for at least a minute. The cDNA Synthesis Mix was prepared. The mix of cDNA contained 2 µl of 10X RT buffer, 4 µl of 25 mM $MgCl_2$, 2 µl of 0,1 M DTT, and 1 µl of Superscript® III RT (200U/ µl). Each RNA mixture was added by 10 µl of cDNA Synthesis Mix, then followed by the incubation process at 50°C, for 50 minutes. After that, reactions were terminated at 85°C for 5 minutes, then immediately chill on ice. Then, 1 µl of RNAse H was collected to each tube and incubated at 37°C for 20 minutes. Products of First Strand cDNA were stored at -20°C.

3) PCR amplification for CHIKV Detection

PCR amplification was performed using My Taq™ HS Red Mix (Bioline, Taunton, MA, USA) Cat. No. BIO-25047, with primer pairs of chikungunya virus, such as forward primer was CHIK 1 (5' - ACC GGC GTC TAC CCA TTC ATG T – 3') 330 bp and reverse primer was CHIK 2 (5' - GGG CGG GTA GTC CAT GTT GTA GA - 3') 330 bp (Yuniarti, 2011). Mix PCR was mixed in a 0,2 ml microtube, with total per reaction is 25 µl contained 12,5 µl of My Taq HS Red Mix 2x, 1 µl of CHIK-1 20 µM (Forward Primer), 1 µl of 20 µM CHIK-2 (Reverse Primer), 1 µl of 100 ng template (cDNA), and 9,5 µl of ddH_2O. Those components were mixed gently and spinned down, then PCR mix was put in thermal cycler with program as follows: (i) One cycle of pre-denaturation at 95°C for a minute; (ii) 35 cycles of amplification step: denaturation at 95°C for 15 seconds, annealing at 60°C for 15 seconds, extension at 72°C for 10 seconds; (iii) A cycle of post-extension at 72°C for 5 minutes.

4) PCR amplification for DENV Detection

Detection of Dengue virus was conducted using My Taq™ HS Red Mix (Bioline, Taunton, MA, USA) Cat. No. BIO-25047, with universal primer pairs of dengue virus (D1 and D2) as first step PCR method continuation, followed by nested-PCR as the second step with universal primer (D1) and serotype-specific primer (TS1, TS2, TS3, TS4). Primer sequences that used were synthesized by Integrated DNA Technologies (IDT, USA). The Oligonucleotide Primers used in RT-PCR for DENV Detection were D1 (5'-TCA ATA TGC TGA AAC GCG CGA GAA ACC G-3'), D2 (5'-TTG CAC CAA CAG TCA ATG TCT TCA GGT TC -3'), TS1 (5'-CGT CTC AGT GAT CCG GGG G-3'), TS2 (5'-CGC CAC AAG GGC CAT GAA CAG-3'), TS3 (5'-TAA CAT CAT CAT GAG ACA GAG C-3'), TS4 (5'-CTC TGT TGT CTT AAA CAA GAG A-3').

The product of cDNA synthesis was used as a template for the PCR method. Mix PCR was mixed in a 0,2 ml microtube, with total per reaction is 25 µl contained 12,5 µl of My Taq HS Red Mix 2x, 1 µl of 20 µM D1 (Forward Primer), 1 µl of 20 µM D2 (Reverse Primer), 1 µl of 100 ng template (cDNA), and 9,5 µl of ddH_2O. Those components were mixed and spinned down, then PCR mix was put in thermal cycler with program as follows: (i) One cycle of pre-denaturation at 95°C for a minute; (ii) 35 cycles of amplification step: denaturation at 95°C for 15 seconds, annealing at 65°C for 15 seconds, extension at 72°C for 10 seconds; (iii) One cycle of post-extension at 72°C for 5 minutes. This PCR product (first step) was directly used for nested-PCR.

Nested – PCR was performed by using the product of PCR from the first step above as a template. Mix PCR was mixed in a 0,2 ml microtube, total per reaction is 25 µl, which contains reagents such as 12,5 µl of My Taq HS Red Mix 2x, 1 µl of 20 µM D1 (Forward Primer), 1 µl of 20 µM TS 1/TS 2/TS3/TS4 (Reverse Primer), 1 µl of 100 ng template (Product of first-step PCR), and 9,5 µl of ddH_2O. Those components were mixed and spinned down, then PCR mix was put in thermal cycler with program as follows: (i) One cycle of pre-denaturation at 95°C for a minute; (ii) 35 cycles of amplification step: denaturation at 95°C for 15 seconds, annealing at 55°C for 15 seconds, extension at 72°C for 10 seconds; (iii) One cycle of post-extension at 72°C for 5 minutes. After PCR was performed, PCR products were confirmed by electrophoresis.

5) Electrophoresis

According to Sari et al. (2012) with modification, products of PCR were confirmed by electrophoresis with 1,5 % agarose gel in TAE

solution buffer. Agarose powder as much as 0,6 grams is diluted with TAE buffer 1x until the volume becomes 40 ml. SYBER Green 5 µl is added, then mixed and homogenized using microwave until the color becomes transparent. Liquid agarose is stored at room temperature for ± 5 minutes, then the agarose gel is poured into the tray and cooled down until solidified. Solidified gel agarose with tray is placed into a chamber then TAE buffer 1x is filled into the chamber. RT- PCR products, as much as 10 µl was pipetted into each well of gel. Electrophoresis was run at 100 V, 400 mA for 50 minutes.

The electrophoresis result was interpreted by UV transilluminator in order to know the band of Dengue and Chikungunya virus. If *Ae.aegypti* is positively infected by DEN-1 virus, the band position is 482 bp, DEN-2 virus band position is 119 bp, DEN-3 virus band position is 290 bp, DEN-4 virus band position is 392 bp (Sari et al., 2012), CHIK virus band position is 330 bp (Yuniarti, 2011). Results were analyzed descriptively by intepreting virus serotype based on gene band appearance using UV transilluminator.

RESULTS AND DISCUSSION

The survey of adult *Ae.aegypti* was followed by molecular detection in order to know the transmission of CHIKV and DENV by their vector. Adult *Ae.aegypti* mosquitoes were collected using BG-Sentinel Traps and put them into microtube for the process of RNA extraction. The number of sample that used for this molecular detection were two samples (pools) for Sokaraja region and each pool includes 20 - 25 mosquitoes. It is in accordance with the research of Sari et al. (2012), which collected 20 mosquitoes each pool to detected Dengue virus using RT-PCR. Detection of DENV in this research was using Two-steps RT-PCR, where RNA of Dengue virus was converted into cDNA first. Product of cDNA then was amplified by RT-PCR that utilizes two universal primers (D1 and D2) with 511 bp amplicon size and followed by nested PCR using TS1-4 primer to know the specific serotype of Dengue virus (Organji *et al*., 2017). The result of dengue virus detection after visualized on agarose 1,5% by using transilluminator is shown in Figure 1.

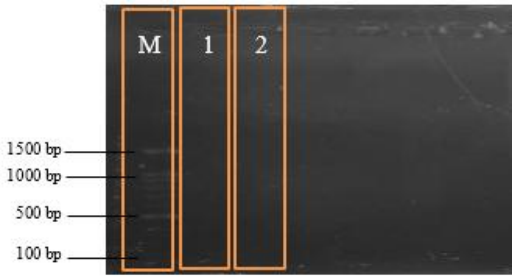

Figure 1. The visualization result of Dengue virus using RT-PCR method on agarose gel 1,5%.

Note: (M) DNA marker 100 bp; (1) Sample 1 of *Ae.aegypti* mosquito collected from Sokaraja region; (2) Sample 2 of *Ae.aegypti* mosquito collected from Sokaraja region

The result of dengue virus detection based on Figure 1 shows that *Ae.aegypti* mosquito which collected in Sokaraja are indicated as negative of Dengue virus serotype DEN 1-4. It is evidenced by the absence of band on the agarose gel as the amplification result. According to Sari et al. (2017), the absence of a band is caused by several factors, such as the limited number of mosquito samples and the low amount of virus titer in the mosquito body, thus it can not be detected using RT-PCR. Moreover, the negative results also can be caused by the status of mosquitoes that distributed in Sokaraja region were not carrying;' any serotype of Dengue virus, thereby when those mosquitoes were detected using RT-PCR, there was no template of DNA virus which can be amplified. It can be estimated that the sufferer of Dengue Fever in Sokaraja region is infested by mosquito from other regions besides Sokaraja. Thus, alteration of the serotype distribution of Dengue virus should be monitored continuously (WHO, 2012). Information about the distribution of dengue virus serotype provides an overview of the level of disease severity, while information related to viral genotypes will provide an overview of Dengue virus that can be used to know where the virus is originated (Ooi & Gubler, 2008).

Detection of Chikungunya virus from *Ae.aegypti* mosquito as a vector was conducted by using RT-PCR. This PCR method is commonly used for Chikungunya detection, according to the research of Pesko et al. (2009), used qRT-PCR to detect *Ae.aegypti* as potential transmitting in Florida, USA. A total of 45 adult *Ae.aegypti* mosquito were obtained in Sokaraja region. All of those samples then pooled, each pool consists of 20 -25 mosquitoes. The Figure below shows the result of Chikungunya virus detection using RT-PCR

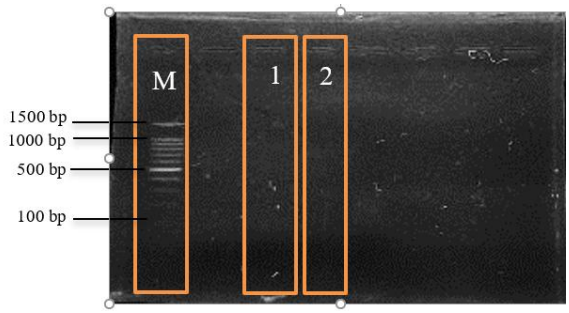

Figure 2. The visualization result of Chikungunya virus using RT-PCR method on agarose gel 1,5%.

Note: (M) DNA marker 100 bp; (1) Sample 1 of *Ae.aegypti* mosquito collected from Sokaraja region; (2) Sample 2 of *Ae.aegypti* mosquito collected from Sokaraja region

According to Figure 2, the visualization of Chikungunya virus detection on agarose gel showed a negative result. The absence of CHIKV band as theproduct of amplification can be caused by several factors, are there is no chikungunya virus in the body of *Ae.aegypti* which collected in Sokaraja and the limited number of *Ae.aegypti* mosquito which carried Chikungunya virus. That mosquito with CHIKV was difficult to be obtained, it is regarding the chikungunya cases is not outbreaking in Sokaraja region. Thereby even though the template is being amplified using RT-PCR, the band still can not be visible (Yuniarti, 2011)..

CONCLUSION

Molecular detection of DENV and CHIKV of mosquitoes which collected from Sokaraja region (Karangrau, Karangnanas, Kalikidang, and Sokaraja Kulon) showed that these regions are free from Dengue and Chikungunya virus.

REFERENCES

Banyumas Regency Health Department , 2015. *Health Profile of Banyumas Regency 2015.* Banyumas: Banyumas Regency Health Office.

Central Java Health Office, 2017. *Health Profile of Central Java Year 2017.* Semarang: Central Java Health Office.

Farraudière, L., Sonor, F.,Crico, S., Etienne, M., Mousson, L., Hamel, R., Misse, D., Failloux, A.B., Simard, F. & Yebakima, A., 2017. First Detection of Dengue and Chikungunya Viruses in Natural Populations of *Aedes aegypti* in Martinique During The 2013 – 2015 Concomitant Outbreak. *Rev Panam Salud Publica*, Volume 41, pp. 1-3.

Harian Banyumas, 2013. 20 Warga Terserang Chikungunya. *Harian Banyumas*, 26 November, pp. 4-5.

Karyanti, R. M., Uiterwaal, C.S. P. M, Kusriastuti, R., Hadinegoro, S.R., Rovers, M. M., Heesterbeek, H., Hoes, A. W.& Bruijning-Verhage, P., 2014. The Changing Incidence of Dengue Haemorrhagic Fever in Indonesia: A 45 Year Registry Based Analysis. *Infection Disease*, 14(412), pp. 1-7.

Karyanti, R. M., Uiterwaal, C.S. P. M, Kusriastuti, R., Hadinegoro, S.R., Rovers, M. M., Heesterbeek, H., Hoes, A. W.& Bruijning-Verhage, P., 2014. The Changing Incidence of Dengue Haemorrhagic Fever in Indonesia: A 45 Year Registry Based Analysis. *Infection Disease,* 14(412), pp. 1-7.

Kraemer, M. U. Sinka, M.E., Duda, K.A., Mylne, A.Q.N., Shearer, F.M., Barker, C.M., Moore, C.G., Carvalho, R.G., Coelho, G, E., Bortel, W.V., Hendrickx, G., Schaffner, F., Elyazar, I.R.F., Teng, H., Brady, O.J., Messina, J.P., Pigott, D.M., Scott,T.W., Smith, D.L., Wint, G.R.W., Golding, N. & Hay, S.I., 2015. The Global Distribution of The Arbovirus Vectors *Aedes aegypti* and *Ae. albopictus*. *eLife Research Article*, Volume 4, pp. 1-18.

Mahmud, S., Ahmed, S. S. & Hussain, M., 2017. Chikungunya Fever: A Global Burden Including Bangladesh. *International Journal of Scientific Research,* 6(7), pp. 542-546.

Ministry of Health Republic of Indonesia, 2016. Info Datin. *Pusat Data dan Informasi Kementria Kesehatan RI*, 22nd April, pp. 1-10.

Ministry of Health Republic of Indonesia, 2016. Info Datin. *Pusat Data dan Informasi Kementria Kesehatan RI*, 22nd April, pp. 1-10.

Muktar, Y., Tamerat , N. & Shewafer, A., 2016. *Aedes aegypti* as a Vector of Flavivirus. *Journal of Tropical Diseases*, 4(5), pp. 1-7.

Nasir, S. Jabeen, F., Abbas, S., Nasir, I.& Debboun, M., 2017. Effect of Climatic Conditions and Water Bodies on Population Dynamics of the Dengue Vector, *Aedes aegypti* (Diptera: Culicidae). *Journal Arthropod-Borne Dis*, 11(1), pp. 50-59.

Ooi, EE and Gubler, DJ. 2008. Dengue in Southeast Asia: Epidemiological Characteristics and Strategic Challenges in Disease Prevention. *Artigo*, 25(1), pp.115–124

Pesko K, Westbrook CJ, Mores CN, Lounibos LP, Reiskind MH. Effects of infectious virus dose and bloodmeal delivery method on susceptibility of *Aedes aegypti* and *Aedes albopictus* to Chikungunya Virus. Journal Medical Entomology. 2009; 46(2):395 –399

Pratama , A. D. & Pawenang, E. T., 2017. Analisis Faktor Intrinsik dan Ekstrinsik Kejadian Penyakit. *Higeia Journal of Public Health Research and Development,* 1(3), pp. 11-20.

Sari, T. F., Joharina, A. S. & Anggraeni, Y. M., 2012. *Identifikasi Serotipe Virus Dengue Pada Nyamuk Aedes aegypti dan Aedes albopictus di Kota Salatiga dengan Metode RT-PCR.* Salatiga: Balai Besar Penelitiana dan Pengembangan Vektor dan Reservoir Penyakit Salatiga.

Sunaryo & Pramestuti, N., 2014. Aedes aegypti Surveillance in Endemic Area of Dengue Haemorrhagic Fever. *Jurnal Kesehatan Masyarakat Nasional*, 8(8), pp. 423-429.

Sus, 2015. 36 Desa/ Kelurahan Endemis DBD. *Radar Banyumas*, 5 December.

Susanti, L., Yuniarti, R. & Trapsilowati, W., 2014. Risk Factors of Chikungunya In Boyolali District, Central Java Province. *BUl. Penelitian Kesehatan*, 42(1), pp. 1-14

Tut, 2013. *Banyumas News.* [Online] Available at: https://banyumasnews.com [Accessed 24 January 2019].

Wahyono, T. Y., Nealon, J., Beucher, S., Prayitno, A., Moureau, A., Nawawi, S., Thabrany, H.& Nadjib, M., 2017. Indonesian Dengue Burden Estimates: Review of Evidence by An Expert Panel. *Epidemiol. Infect*, Volume 145, pp. 2324-2329.

WHO, 2012. *Global Strategy for Dengue Prevention and Control 2012-2020.* Geneva: World Health Organization.

Yuniarti, R. A., 2011. *Studi Rekonfirmasi Penyakit Chikungunya di Daerah Endemis Jawa Tengah*, Salatiga: B2P2VRP.

Zeller, H., Bortel, W. V. & Sudre, B., 2016. Chikungunya: Its History in Africa and Asia and Its Spread to New Regions in 2013-2014. *Journal of Infectious Disease*, 214(5), pp. 436-440.