Indonesian Journal of
Tropical and Infectious Disease
Vol. 7 No. 3 September–December 2018

Research Report

PRELIMINARY STUDY OF *WUCHERERIA BANCROFTI* L3 LARVAE DETECTION IN *CULEX QUINQUEFASCIATUS AS* VECTOR POTENTIAL OF FILARIASIS IN ENDEMIC AREA OF SOUTH TANGERANG, BY UTILIZING PCR ASSAY FOR L3-ACTIVATED CUTICLIN TRANSCRIPT *m*RNA GENE AND TPH-1 GENE

Silvia Fitrina Nasution[1a], Chris Adhiyanto[1], Evi Indahwati[1]
[1] Faculty of Medicine, Islamic University of Syarif Hidayatullah Jakarta
[a] Corresponding Author: silvia.nasution@gmail.com

ABSTRACT

South Tangerang district is an endemic area for Wuchereria bancrofti filariasis with a prevalence rate of microfilaria (mf) at a range of 1 - 2.4% in 2008-2009. Culex quinquefasciatus plays an important role as the major vector of transmission for the parasite. It remains a problem on how to determine that the mosquitoe roles as a vector or disease transmitter when there is no evidence of filarial parasite larvae 3 (L3) by the microscopic examination. In assessing the transmission risk of the filarial parasite, a DNA-based detection method was carried out to specifically detect the presence of W. bancrofti infective L3 larvae in the mosquitoe. The Polymerase Chain Reaction (PCR) was performed to detect a specific DNA obtained from W. bancrofti L3 larvae in a very low number or low antigen titer. The assay was purposed as preliminary study to detect the presence of L3 filarial of W.bancrofti in Cx. quinquefasciatus by utilizing the expression of L3-activated cuticlin transcript mRNA gene and tph-1 gene. The result of PCR based analysis of mosquitoes collected from the suggested area showed that there is a low but detectable number of L3 infected mosquito with W. bancrofti. Among the 18 isolated DNA samples of mosquitoes, we found 7 positive samples (38.89%) with the presence of filarial larvae DNA expressing L3-activated cuticlin transcript mRNA and tph-1 genes, which determined as 123 bp for Wb-cut-1.2 and 153bp for tph-1. In contrast by microscopic result, we found no evidence of L3 larvae of the parasite in the mosquitoe's dissecting samples. The PCR assay in our study was proven sensitive to detect the presence of Wb-L3 filarial larvae in Cx. quinquefasciatus

Keywords: *W. bancrofti, Cx. quinquefasciatus, PCR, L3-activated-cuticlin transcript mRNA, Wb-cut-1.2, gen tph-1*

ABSTRAK

Kabupaten Tangerang Selatan merupakan daerah endemis filariasis Wuchereria bancrofti dengan prevalensi mikrofilaria berkisar 1-2.4% yang dilaporkan pada tahun 2008-2009. Culex quinquefasciatus berperan penting sebagai vektor utama penyebaran parasit tersebut. Permasalahan yang masih ada yaitu bagaimana menentukan bahwa nyamuk tersebut berpotensi sebagai vektor atau penular penyakit bila tidak ditemukan bukti adanya larva stadium 3 parasit filaria (L3) secara mikroskopis. Untuk mengetahui risiko penularan dari parasit filaria tersebut, maka dilakukan suatu metode deteksi berbasis DNA yang bertujuan untuk mendeteksi secara spesifik keberadaan larva 3 infektif dari W. bancrofti di dalam tubuh nyamuk. Metode yang dilakukan adalah dengan menggunakan Polymerase Chain Reaction (PCR) untuk mendeteksi DNA spesifik dari L3 W. Bancrofti dalam jumlah ataupun titer antigen yang sangat rendah. Pemeriksaan ini bertujuan sebagai study pendahuluan untuk mengetahui adanya L3 filaria W. bancrofti di dalam nyamuk Cx. quinquefasciatus dengan menggunakan ekspresi gen L3-activated cuticlin transcript mRNA dan tph-1 secara PCR. Hasil analisa PCR terhadap nyamuk yang diperoleh dari wilayah tersebut menunjukkan masih ditemukannya nyamuk yang terinfeksi oleh larva L3 W. bancrofti dalam jumlah yang rendah. Di antara 18 sampel isolat DNA nyamuk, terdapat 7 sampel yang positif (38.89%) mengandung DNA larva filaria yang mengekspresikan gen L3-activated cuticlin transcript mRNA dan gen tph-1 dengan genom sebesar 123 bp pada Wb-cut-1.2 dan 153 bp pada tph-1. Sebaliknya, hasil pemeriksaan mikroskopis tidak ditemukan adanya larva L3 dari parasit tersebut

di dalam sampel bedah nyamuk. Uji PCR pada penelitian ini terbukti sensitif dapat mendeteksi keberadaan L3 filaria W. bancrofti dalam tubuh nyamuk Cx. quinquefasciatus

Kata kunci: *W. bancrofti, Cx. quinquefasciatus, PCR, L3-activated-cuticlin transcript mRNA, Wb-cut-1.2, gen tph-1*

INTRODUCTION

Filariasis has been widely distributed in almost all of Indonesian regions, and in some areas the endemicity rate is relatively high. The endemic areas of *Wuchereria bancrofti* are categorized into rural and urban type based on the vectors. Rural type of *Wuchereria* is mainly found in Papua and Nusa Tenggara with *Anopheles, Culex* and *Aedes* as the vectors; while urban type is found in Jakarta, Bekasi, Tangerang, Semarang, Pekalongan and Lebak in slum and densely populated area that has a great amount of dirty puddle with *Culex quinquefasciatus* as the main vector.[1] Study by Nasution SF, 2015 has reported that *W.bancrofti* was the major filarial parasite of the disease in this endemic area of South Tangerang.[2]

The life cycle of the parasite requires two hosts: the mosquito vector and the human host. Part of the developmental life cycle of the parasite occurs in the mosquito and the other part in the human host. The parasite develops through four stages in the mosquito (microfilaria, L1,L2, and L3), only the last of which is infectious to humans. The third larval stage (L3) is the infective stage that initiates human infections when infective mosquitoes bite humans. Therefore, it can be said that the mosquito has infective nature.[3] The development of L3 stage in mosquitoes ranges about 2 weeks. It causes a problem to find L3 in the body circulation before it reaches such age in wildlife.[4]

Studies on the prevalence of *W.bancrofti* in mosquitoes have traditionally relied on manual dissection and microscopic examination.[5] Dissection is not practical nor is it sensitive enough for detecting and measuring mosquito infection and infectivity when rates are very low following MDA.[3] Another problem is to detect the parasite circulation in a very low number or antigen titer of L3 infective stage in the mosquitoes. Other diagnostic tests have recently been developed that include the polymerase chain reaction (PCR)-based detection of *W. bancrofti* DNA in mosquitoes and human blood sample. These advances have allowed sampling during the day for humans and high through put analysis for both human and vector samples, and in turn increased the sensitivity of LF diagnosis.[5]

The PCR based detection of *W. bancrofti* DNA in mosquitoes provides an indirect measure of filarial infection rates in human populations, but is not to purpose a measure of transmission. The reason is because PCR detects DNA from all filarial larvae stages in mosquitoes indistinctively. Therefore measures "infection" in mosquitoes and not "infectivity" as not all microfilariae (Mf) ingested by mosquitoes survive and develop into infective L3 stage. To directly measure transmission potential, the presence of L3 stage in the vector must be evaluated.[3]

Detection by utilizing PCR assay, which is specific for infective *W. bancrofti* L3 larvae, is significantly benefit to evaluate the successful program of filariasis vector, by measuring infection rate post Mass Drug Administration (MDA). Furthermore, it will provide an important information to end the MDA program in suggested area. In advance, it allows to detect potency of re-infection at the final program of MDA. Ultimately, it will improve our knowledge on the pathogenesis of Limphatic Filariasis (LF) as well as the complexity of the vectors which have transmission potential in various endemic areas. In this case, the detection of L3 stage can be used to identify and to confirm the species of mosquitoes that has a role as vector in LF transmission.[3]

MATERIAL AND METHOD

The study was designed in cross-sectional with cluster sampling method in suggested area of filariasis in Kampung Sawah, Tangerang Selatan District. We collected mosquito samples of *Cx. quinquefasciatus* from selected cluster houses of individual with clinical filariasis as well as serological positive in accordance to Nasution SF, 2015; and also some neighbors living nearby. The sample size was about 100 adult female mosquitoes with human blood fed and actively moving during collection (inclusion criteria).

This study was also conducted according to previously published protocols by Laney SJ., et al. 2010. In this work, we identified *W. bancrofti* L3-activated gene targets (mRNA transcripts first expressed in infective stage larvae) and developed a WbL3-detection assay that can determine the presence or absence of the infective stage of *W. bancrofti* in mosquito vectors using reverse transcriptase polymerase chain reaction (RT-PCR). This multiplex assay can simultaneously detect 'any stage' of the parasite in pools of mosquitoes yielding both infection and transmission potential data from the same samples. Most importantly, this new WbL3-diagnostic tool has the potential to improve our understanding of the impact of MDA on LF transmission.[3]

Mosquito Collection and Dissection

Briefly, we demonstrated the responden how to collect samples from indoor resting mosquitoes by using some materials such as inspirator tube of mosquitoe trap, mosquito cage, and sugar feeding. We selectedly collected all female blood fed of indoor resting mosquitoes when hung on the wall or clothes by trapping and sucking them into the inspirator, to readily replaced them into the

mosquito cage. Each cage was placed by 10 blood fed mosquitoes and put some soaked cotton by 10% of sugar solution mixed with vitamin C on top of the cage, to feed the mosquitoes and preserve them before dissection.

In order to preserve the parasites and to develop them growth into L3 stage, we collected & reared the mosquitoes in an insectary room at 27°C±2°C at 80% relative humidity, and daily feed them by 10% sugar solution for 10-14 days post blood feeding. Unfortunately, no mosquitoes remained alive before day 10. We directly dissected them under microscope, soon as they knocked down or paralysed. Each mosquito was placed on a slide and the head, thorax, and abdomen were macerated separately in a drop of saline and examined under a compound microscope at 100x for larvae.[6] Result of the dissections revealed no *W. bancrofti* larval stages in the head, thorax or abdomen of the examined mosquitoes. They were then put into a microcentrifuge tube for the next process of DNA purification and PCR.

Identification of Gene Expression

Reverse transcriptase PCR (RT-PCR) was performed to detect the expression of L3-activated cuticlin transcript mRNA of the filarial. Conventional and real-time multiplex RT-PCR employing L3-activated cuticlin transcript (RNA transcripts, which produced by the L3 cuticula larvae) were conducted to detect the profile of L3 *W. bancrofti* and tph-1 gene expressions for all stages of larvae L1, L2, L3 and Mf. Identification of *W. bancrofti* L3 larvae was examined by tracing cDNA sequence in dbEST database index in NCBI BLASTN program. Results of identification on *W. bancrofti* L3 cuticula sequences are categorized into 3 DNA clusters and labeled as cut-1.0, cut-1.1 and cut-1.2 using SeqMan Program of the Lasergene Suite (DNAStar, Inc.).[2]

Wuchereria bancrofti cuticle-related genes were selected for expression profile screening through bioinformatics searches and screened as potential diagnostic target genes for L3 detection in mosquitoes. The DNA sequence as a primer produced by PCR is determined as 123 bp[3], 129 bp[7] and 188 bp[9] of *W. bancrofti* DNA fragment. In this study, the *W. bancrofti* cut-1.2 cuticlin gene was selected as the diagnostic target for the L3-detection assay. The Wb-cut-1.2 primer was identified as *W. bancrofti* L3 stage larvae cuticlin which emerges L3 in 9 dPBM = days post blood meal (collection point after mosquitoes were fed on infected blood).[3]

The DNA allows to detect the presence of *W. bancrofti* in *Cx. quinquefasciatus.* The specificity of PCR product was confirmed by sequencing and slot blot hybridization assay. High sensitivity was found for *W. bancrofti* by detecting parasitaemia up to the level of one microfilaria per reaction.[7]

DNA Extraction and Isolation

The DNA extraction and isolation from mosquitoes was performed by Chelex methods according to previously described by Santoso 2015. Briefly, we separated thorax, head, and proboscis from abdoment to dissolve with 100 µl ddH_2O and grinded with a pestle in a tube. The suspension was then made by adding 1 ml of 0,5% saponin + PBS. Following the addition, incubate it in 4°C for 3 hours; then centrifuge it in 12.000 rpm for 10 minutes; throw out the supernatant; Add 1 ml PBS 1x; continously centrifuge it in 12.000 rpm for 5 minutes; then throw out the supernatant; mix with 100 µl ddH_2O and 50 µl 20% chelex 100; heat it into 37°C for 10 minutes; vortex for 5 minutes; the supernatant was transferred to a sterile tube, and fill it up to 100 µl; then incubate the gDNA in freezer by temperature of -20°C.[8]

DNA extraction and PCR process were conducted by using a pool sized 50–100 µl filled by salivary glands of mosquitoes. Afterwards, DNA purification was carried out using *Geneclean* (Bios 101).[9]

DNA Amplification

In preliminary study, we made suspension of mosquitoes DNA and mix with the DNA of L3 filarial and 25 mL of water. The suspension was then diluted starting with 1:5 to 1:625. The procedure of DNA isolation was as follow:[9]

1. **DNA amplification of L3 filaria**
   An optimum DNA amplification of L3 filarial will constantly established if 1 mL of DNA made in 1:625 dilution. When 10 mL DNA of a total 25 mL PCR product was visualized in 1% ethidium bromide stain and loaded in agarose gel, then 188 base pairs (188 bp) of *W. bancrofti* DNA should be appeared. Furthermore, the preparation for Reverse Transcriptase PCR (RT-PCR) test was performed using the bioinformatics protocol.[6,9]
   Wb-cut-1.2 primers for conventional RT-PCR amplified a 123 base pair (bp) product from WbL3 RNA, while primers for tph-1 amplified a 153 bp fragment from all stages of the parasite.
2. **Conventional One-step RT-PCR Wb-L3 Assay**
   Conventional multiplex RT-PCR reactions were performed based on a standard condition of one-step RT-PCR kit (Qiagen, Inc.) (Table 1) in 600 nM of each primer (cut-1.2 forward primer #1938 and reverse primer #1939, as well as tph-1 forward primer #1054 and reverse primer #1059) and 1 mL RNA template in 25 mL total volume.[3]

Table 1. Formula procedure of PCR reagent

Components	Volume (µL)	Wb cut 1.2	tph-1 (B)
PCR grade water	5.6	5.6	
2xKAPA syber fast (mastermix)	10	10	
50xROX	0.4	0.4	
10 mMdUTP	0.5	0.5	
10 µM forward primer	0.5	0.5	
10 µM reverse primer	0.5	0.5	
50x KAPA RT mix	0.5	0.5	
Template RNA	2.0	2.0	
Total	20	20	

Amplification condition of PCR cycle in the procedure was 50°C for 30 minutes, 95°C for 15 minutes and 57°C for 5 minutes that proceed in 1x cycle. Continously proceed in 72°C for 90 seconds, 94°C for 45 seconds, 57°C for 45 second in 40x cycles. Final extention was set in 72°C for 10 minutes.

3. **Two Steps of PCR reverse transcription**
   The following RNAs are appropriate for highly efficient reverse transcription: (by TOYOBO Bio-Technology).[10]
   (1) Total RNA
   Total RNA usually contains 1-2% mRNA that was directly used as PCR template kit. RNA was prepared with AGPC (Acid Guanidium-Phenol-Chloroform) or utilizing column which coated by genomic DNA; therefore, the total RNA should be equipped with DNase-1 prior to transcription.
   (2) Poly $(A)^+$ RNA (mRNA)
   Poly $(A)^+$ RNA has ability to detect RNA expressions with low concentration. However, Poly $(A)^+$ RNA should be carefully handled since it is more sensitive against RNase compared to total RNA.

Reverse transcription iScript™ cDNA synthesis Kit

Prepare all of necessary reaction solutions and measure RNA concentration precisely before making a mastermix. The mastermix proceed as follows (Table 2):[11]

Table 2. Master Mix Procedure

Kit contents	Volume (µL)			
	Sample No.	15	8	10
5X iScript Reaction Mix4	4	4	4	
iScript Reverse Transcriptase1	1	1	1	
Nuclease free water X	12	8	5	
RNA template (100 fg – 1 µg total RNA)X	3	7	10	
Total	20	20	20	

The master mix was incubated for 5 minutes at 20°C, then continue the process for 30 minutes at 42°C, and heat it for additional 5 minute incubation at 85°C. As final step, store the reacted solution at 4°C before PCR.[11] When the incubation process is completed, the cDNA solution is ready for PCR analysis or other analysis. For the next step, we conducted electrophoresis for about 30 minutes to 1 hour period of time. Afterwards, 100 volt voltage and 20°C was set.

RESULT AND DISCUSSION

Microscopic Results

Among 100 samples of blood fed mosquitoes that we collected from field, there were only about 50 mosquitoes remained alive after one day reared at insectarium lab. Following the next 5 days, only 25 mosquitoes alive and we continue preserved them with sugar feeding.

On the day 5 to 7, we dissected about 25 knock down mosquitoes and we found no stages of L1, L2, L3 larvae either in their thorax, head, nor proboscis by microscopic result.

PCR Results

The conventional one step RT-PCR (Qiagen, Inc.) utilized Wb-cut-1.2 and tph-1 primers shows the following results:

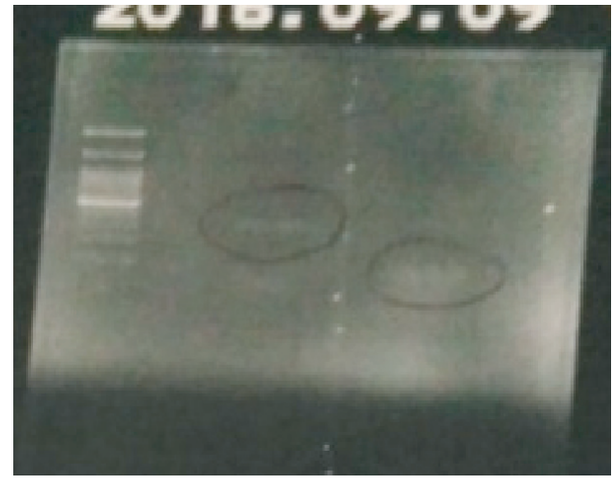

Figure 1. One step PCR result of individual sample

Figure 1 is showed 2 bands of both samples which utilized Wb-cut-1.2 and tph-1 primers. The Amplification of L3-activated Wb-cut-1.2 transcript appeared on genome with molecular weight (MW) of 123 bp and tph-1 transcript on MW of 153bp.

Meanwhile, the same primers were applied for 7 different samples and showed the following result:

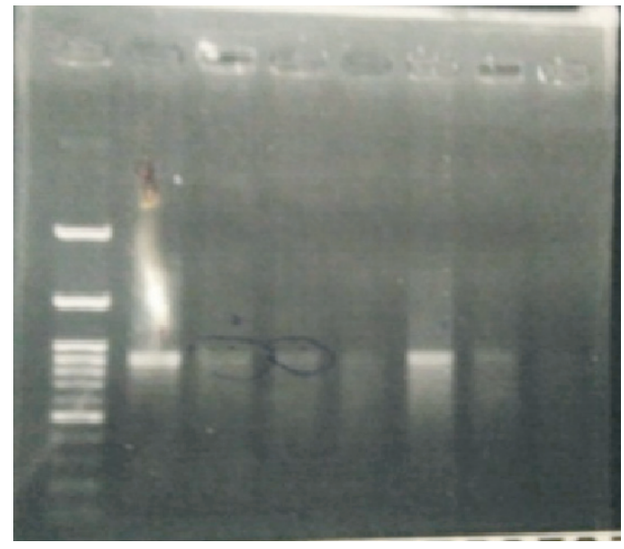

Figure 2. One step PCR result for 7 samples

Figure 2 is showed the formation of 7 bands of RNA samples with different thickness. There are 2 samples with significantly clear band, in which DNA amplification was carried out for both samples, then proceed to make copy of the DNA. The DNA copy was assayed by utilizing 2-steps PCR to generate genome sequence with thick band. The cDNA assay appeared as seen in Figure 3.

Figure 3. Two steps PCR result of 2 cDNA samples

In Figure 3, there is no formation of cDNA band, which indicates that both samples showing negative results on the presence of L3-activated cuticlin Wb-cut-1.2 and tph-1.

Conventional diagnosis of LF depends on detection of microfilariae (Mf) in blood specimens, which has low sensitivity and specificity.[12] This will related to the amount of parasite sucked by the mosquitoe. Microscopic examination and manual dissection on detecting parasitic larvae of *W.bancrofti* in mosquitoes have no practical nor sensitive enough for measuring mosquito infection and infectivity when rates are very low following MDA. Another problem is to detect the parasite circulation in a very low number or antigen titer of L3 infective stage in the mosquitoes. In this study, we found that all mosquitoe samples were negative of filarial larvae of all stages (Mf,L1,L2,L3) by dissecting mosquito salivary glands, although we reared the samples in 5, 7, 10 to 14 days post blood meal. We define some possible reasons for such results, as followed: there are no filarial larvae in the blood fed mosquiotes or amicrofilaremic human blood; amount of larvae was very small density or they were destroyed during dissection; the mosquito died earlier before its larvae develop into L3 stage; and poor skill on dissecting mosquito or to identify larvae stage. PCR based detection allows to accommodate this obstacle of microscopic examination. The assay has high sensitivity to detect specific infective *W. bancrofti* L3 larvae in mosquito and is significantly benefit to evaluate the successful program of filariasis vector, by measuring infection rate post Mass Drug Administration (MDA).

Reverse transcriptase PCR (RT-PCR) was performed to detect the expression of L3-activated cuticlin transcript mRNA of the filarial. The L3-activated cuticlin transcript mRNA were utilized to reveal the profile of L3 *W. bancrofti* and tph-1 gene expressions for all stages of larvae L1, L2, L3 and Microfilariae.

Our study result by conventional one-step PCR utilizing the genome has determined significant figure in 7 among 18 (38.89%) samples with RNA concentration ranged between 18.407 and 58.331 ng/μL of mosquitoes. The Amplification of L3-activated **Wb-cut-1.2** transcript appeared on genome with molecular weight (MW) of 123 bp and **tph-1** transcript on MW of 153bp (figure 1 & 2). Similar result was reported by Laney, et al. 2010, that the DNA sequence was expressed by the same primer of Wb-cut-1.2 as 123 bp[3], 129 bp[7] and 188 bp[9] of *W. bancrofti* DNA fragment. The Wb-cut-1.2 primer was identified as *W. bancrofti* L3 stage larvae cuticlin which emerges L3 in 9 dPBM = days post blood meal, on this study.

The DNA copy was assayed by utilizing 2-step PCR in order to generate genome sequence of the samples with thick band. The result showed no formation of cDNA band, which indicates that all samples are negative on the presence of L3-activated cuticlin Wb-cut-1.2 and tph-1. In accordance with this result, we determine some reasonable explanations as followed: lack of density and quality of cDNA to cause poorly detectable by PCR,unoptimized product of amplification, or contaminated PCR product to possibly cause cross-reaction with other contaminant, or other human error during the process.

CONCLUSION

The PCR assay by utilizing the expression of L3-activated cuticlin transcript mRNA gene and tph-1 gene was proven sensitive to detect the presence of Wb-L3 filarial larvae in *Cx. Quinquefasciatus* and all stages of filarial larvae which is not detectable by microscopic.

We suggest to continue the study and to improve the method and sampling in a large number of mosquitoe's population from the endemic areas of filariasis in South Tangerang District.

ACKNOWLEDGEMENT

The study is acknowledged to Research Center of (Pusat Penelitian) UIN Syarif Hidayatullah Jakarta, Indonesia as our study financial support, and to all technical support by my colleagues in Parasitology Department of UIN Syarif Hidayatullah Jakarta.

REFERENCES

1. Prof.-dr-srisasi-gandahusada-parasitologi-kedokteran-edisi-tiga.-intro (1).pdf.
2. Nasution S. Comparative Study of Filarial Detection by Microscopic Examination and Serological Assay Utilizing BMR1 and BMXSP Recombinant Antigens for Evaluation of Filariasis Elimination Program at Kampung Sawah and Pamulang, South Tangerang District, Banten, In. Indones J Trop Infect Dis. 2015 Dec 11;5(6):156.
3. Laney SJ, Ramzy RMR, Helmy HH, Farid HA, Ashour AA, Weil GJ, et al. Detection of Wuchereria bancrofti L3 larvae in mosquitoes: a reverse transcriptase PCR assay evaluating infection and infectivity. PLoS Negl Trop Dis. 2010 Feb 16;4(2):e602.
4. Chai J-Y. Ash & Orihel's Atlas of Human Parasitology (5th ed.). Korean J Parasitol. 2007;45(4):311.
5. Kouassi BL, de Souza DK, Goepogui A, Narh CA, King SA, Mamadou BS, et al. Assessing the presence of Wuchereria bancrofti in vector and human populations from urban communities in Conakry, Guinea. Parasit Vectors. 2015 Sep 26;8:492.
6. Gbakima AA, Appawu MA, Dadzie S, Karikari C, Sackey SO, Baffoe-Wilmot A, et al. Lymphatic filariasis in Ghana: establishing the potential for an urban cycle of transmission. Trop Med Int Heal. 2005 Apr;10(4):387–92.
7. Mishra K, Raj DK, Hazra RK, Dash AP, Supakar PC. The development and evaluation of a single step multiplex PCR method for simultaneous detection of Brugia malayi and Wuchereria bancrofti. Mol Cell Probes. 21(5–6):355–62.
8. Santoso Santoso, Yahya Yahya, Nungki Hapsari Suryaningtyas KSR. Deteksi mikrofilaria Brugia malayi pada nyamuk Mansonia spp dengan pembedahan dan metode PCR di Kabupaten Tanjung Jabung Timur. ASPIRATOR. 2015;7(1):29–35.
9. Furtado AF, Abath FG, Regis L, Gomes YM, Lucena WA, Furtado PB, et al. Improvement and Application of a Polymerase Chain Reaction System for Detection of Wuchereria bancrofti in Culex quinquefasciatus and Human Blood Samples. Mem Inst Oswaldo Cruz. 1997 Jan;92(1):85–6.
10. Aution C. ReverTra Ace ® qPCR RT Master Mix with gDNA Remover. (86).
11. PT. Sciencewerke Indonesia. Instruksi kerja iScriptTM cDNA Synthesis Kit. SWI-FAS-GEN-IK- 1708890. 2016.
12. Nuchprayoon S. Review: DNA-based diagnosis of lymphatic filariasis. Southeast Asian J Trop Med Public Health. 2009;40(5):604–13.