Molecular Detection for Japanese Encephalitis and Chikungunya Virus as Etiology Agent for Dengue-Like Fever Symptoms

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Abstract

Background: Acute fever with dengue-like fever symptoms with headache, rash, arthritis, perifer bleeding like petechiae and rinhorrhea are symptoms that often complained by patient and it may be caused by arboviruses infection. Arboviruses are very endemic in Indonesia, especially dengue virus (DENV), Japanese encephalisitis virus (JEV), and chikungunya virus (CHIKV) that causing similar symptoms. Molecular detection is very important to confirm the etiology for adequate management. **Methods**: This study investigated the etiological agent using one step real time RT reverse transcription polymerase chain reaction (RT PCR) method and SYBR Green I as fluorescent binding dye reporter. The viruses that were investigated were JEV and CHIKV. Primers were designed using online software NCBI. Those primers fulfill the good primer requirements and can be used as material in RT PCR reaction. **Results**: The optimum temperature for all primers were at 60 °C. The detection limit of JEV primer was 4355 copy DNA every reaction. Cross reactivity between all primers with DENV serotype 2 RNA and false positive result using healthy person sample were not found. **Conclusions**: This study has optimized condition for RT PCR protocol that can be used as diagnostic tools for patient with dengue-like fever symptoms.

Keywords: arboviruses, chikungunya virus, dengue japanese encephalisitis virus, reverse transcription polymerase chain reaction

Introduction

In Indonesia, the endemic arboviruses, i.e. DENV, CHIKV, and JEV cause similar symptoms at acute phase. DENV infection is very important in Indonesia, because it endemic annually in Indonesia. The biggest outbreak was in 1998 and 2004 with 79.480 DENV infection cases with more than 800 death.[1,2] Confirmed CHIKV infection had been reported in Indonesia in 1973, and from 2007 to 2012 with 149.52 CHIKV infection without fatal case. The CHIKV infection usually occur where DENV infection also develop. Both infection were transmitted by *Aedes aegypti*.[3] The study from Suharti et al in Semarang, Indonesia found that from 118 patients with suspect DENV infection, 58 were confirmed with DENV and 60s other was not DENV infection. The 60s other were tested for other viruses and 2 case CHIKV infection, 5 Hantavirus infection, and 1 Influenza A infection were reported.[4] Indonesia also endemic with JEV infection and first infection was found in Bali in 1996. The surveillance in

Bali found 86 patients positive JEV infection confirmed by laboratory test from 239 pediatric patient.[5] The gold standard for JEV, DENV, and CHIKV infection is finding the virus, either in virion form or the gene of the virus and followed by detection of antibody against the viruses after five days of fever.[6–8] The strategy to overcome the viral infection in human is identifying and detecting the agent causing the disease, therefore appropriate medication and therapy can be promptly given. Indonesia has really great potency with the spreading of arboviruses because of the climate in this country, in consequences, Indonesia needs establishment of molecular detection for arbovirus infection. The symptoms of this infection is similar like acute fever with rash, arthritis, rinorrhea, petechiae, and headache. The disease could not be distinguished by the symptoms, therefore confirmation test is needed to identify the etiology of the disease. This study was using one step reverse transcriptase real time PCR and SYBR Green I as reporter dye because it provide the time and cost effectiveness. Reverse transcriptase real

time PCR is one of the method to detect the genetic material from the virus and produce the accurate data, therefore this study aimed to develop the molecular detection of JEV and CHIKV by using real time RT PCR.

Methods

Viruses. DENV-2 RNA was used for negative control at real time RT PCR reaction. DENV serotype 2 was cultured at Vero cell in Microbiology Laboratory, Faculty of Medicine, Universitas Indonesia. The study was approved by Ethical Committee of Faculty of Medicine Universitas Indonesia.

Healthy Person Sample. One healthy person sample was used to evaluate the specification of the RT PCR. The sample was collection from Microbiology Clinic Laboratory, Faculty of Medicine, Universitas Indonesia. The criteria of sample was blood of person who had not any symptoms of disease and was checked by NS1 DENV with negative result. The volume of sample was 140 μL plasma.

Primer Design. The primer was designed using online software Primer 3 and NCBI based on CHIKV Bali Isolate (GeneBank MF773561.1) and JEV Isolate JaGar 01 (GeneBank AF069076.1) RNA sequence. Primers were designed based on the specification for primer such as size of primer, size of amplicon, and avoidance from primer dimer. The sequence JEV and CHIKV primers were described in Table 1. The forward and reverse JEV primer consist of 20 bp and produced 136 bp amplicon size. Forward primer annealed at nucleotide 8640 to 8659 and reverse primer annealed to nucleotide 8775 to 8756. The gene target from the primers was NS5 gene that highly conserved at genome of JEV. Primer CHIKV consist of 20 bp for forward or reverse primer and produced 70 bp amplicon size. Forward primer annealed at nucleotide 5917 to 5936 and reverse primer annealed to nucleotide 5986 to 5967. Gene target from the primer was NSP4 gene of CHIKV.

DNA Synthetic Design. This study designed sequence of DNA synthetic for positive control. This DNA was designed based on target sequence of JEV and CHIKV primer. DNA was constructed from target sequence of some viruses including JEV and CHIKV and can be used for positive control because the DNA was similar with the virus target. The target sequences were obtained using blast primer of NCBI online software. The sequence target was chosen form one isolate each viruses and then constructed as DNA synthetic. The size of DNA synthetic was 1047 base pairs. DNA synthetic was prepared by IDT DNA (PT. Genetika Science).

Table 2 describe about position and size of amplicon that produce by JEV and CHIKV primer

Viral RNA Extraction. The RNA sample and RNA DENV was extracted using QIAamp Viral RNA Mini Kit (Qiagen, Japan). Extraction was done in BSC II in BSL 2 laboratory. The extraction steps followed the instruction from the kit. After the extraction, the RNA was aliquoted to 3 tubes with 20 μL volume each tube and stored at -80 °C.

Real Time RT PCR. This study used one step reverse transcription real time PCR. The PCR reagent used in this study was NexQ 7000 (Bioneer, Korea). The PCR cycle of this reaction was reverse transcription 55 °C for 30 minutes and initial denaturation 95 °C for 10 minutes followed by 40 cycling of denaturation 95 °C for 30 seconds and annealing 56 °C to 62 °C for 1 minute, done in Biorad iQ5 (Biorad, US). The RT PCR reaction used SYBR Green I as fluorescent binding dye reporter, therefore the melting cycle was activated for this methods. Synthetic DNA was used for universal positive control (Figure 1).

Sensitivity. The sensitivity of the test was determined by detection limit of RT PCR used diluted synthetic DNA as template for the reaction. Ten fold serial dilutions from synthetic DNA were used to determine the sensitivity of the assay. Concentration of DNA synthetic in this test was at 1 pg, 10^{-2} pg 10^{-4} pg, 10^{-5} pg, and 10^{-6} pg, which is equal to 8.71×10^7, 8.71×10^5, 8.71×10^3, 8.71×10^2, 87.1 copies DNA.

Specificity. The specificity from JEV and CHIKV primers was evaluated using RNA from DENV-2 and healthy person plasma.

Table 1. Spesification JEV and CHIKV Primer

Primer	Position
CHIKV Forward	5917 - 5936
CHIKV Reverse	5986 - 5967
JEV Forward	8640 - 8659
JEV Reverse	8775 - 8756

Table 2. Position (nt) of CHIKV and JEV Primer in Synthethic DNA sequence

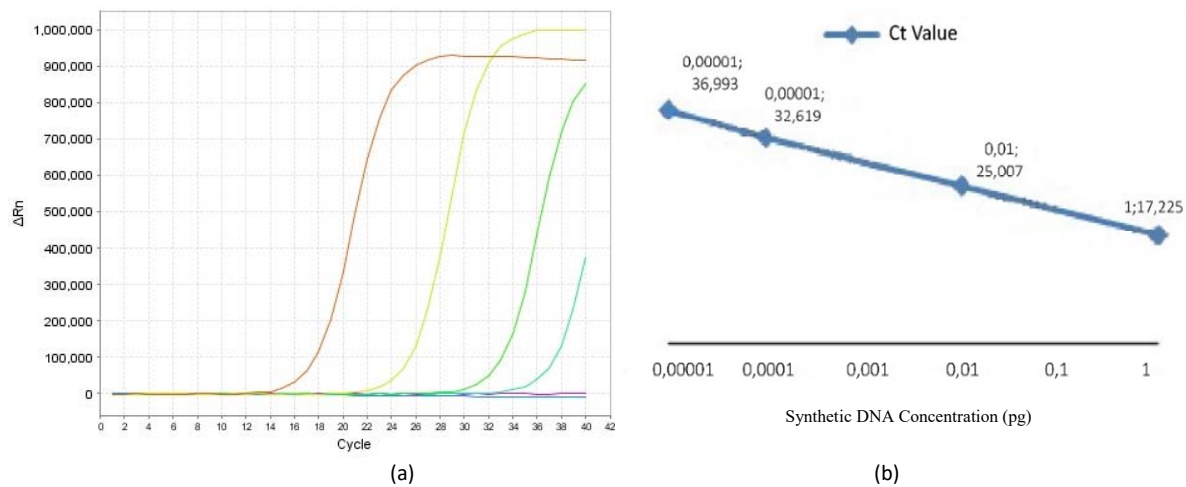| Primer	Primer Position	Amplicon Size
CHIKV F	783 - 202	70
CHIKV R	833 - 852	
JEV F	853 - 872	136
JEV R	1028 - 1047	

Figure 1. (a) Amplication plot; (b) Limit of detection JEV primer curve

Results

Optimum Annealing Temperature. Optimum annealing temperature for JEV and CHIKV primer was determined using gradient temperature; 56 °C, 56.8 °C, 58.1 °C, 59.9 °C, and 62.4 °C. The optimization used 100 pg synthetic DNA as template. JEV primers produced Ct value between 7 and 8 at temperatures 56 °C to 59.9 °C and had higher Ct (12.1) with annealing temperature of 62.4 °C. Melting temperature for JEV primers at all tested annealing temperature had similar value (88.5 °C to 89 °C). The result for CHIKV annealing temperature at 62.4 °C was negative, and positive at 56 °C to 59.9 °C. (Fig 2). There was negative result at 10^{-6} pg or 433.5 copy DNA per reaction.

Specificity of JEV and CHIKV Primer. To verify the specificity of the primers, we used healthy person plasma and DENV-2 RNA as negative control. The

Between four gradient annealing temperature that have positive result, only one annealing temperature at 59.9 °C made one peak at melting temperature while the three others (56 °C, 56.8 °C, and 58.1 °C) produced two peaks melting temperature. The results of Ct values from the four annealing temperature were similar (Table 3).

Sensitivity of JEV Primers. The detection limit was used to determine the sensitivity of JEV primers. This study used five ten fold dilution of synthetic DNA concentration. The minimum concentration that can be detected by JEV primer was at 10^{-5} pg that equal to 4355 copy DNA per reaction with Ct value 36.993 results showed that JEV primers did not react with DENV-2 RNA and healthy person sample (Table 4). CHIKV primers detected some amplification with DENV-2 RNA as template but with melting temperature different from the positive control (Table 4).

Table 3. Optimization of gradient annealing temperature

Temperatures	JEV		CHIKV	
	Ct	**Tm**	**Ct**	**Tm**
56 °C	7.77	89 °C	6.69	85.5 °C; 89.5 °C
56.8 °C	7.22	88.5 °C	7.61	85.5 °C; 89 °C
58.1 °C	7.81	88.5 °C	8.29	85.5 °C; 89 °C
59.9 °C	8.63	89 °C	9.6	89 °C
62.4 °C	12.10	88.5 °C	N/A	89 °C

N/A: negative; Ct: Treshold Cycle; Tm: Melting temperature

Forward Primer   Reverse Primer

(a)

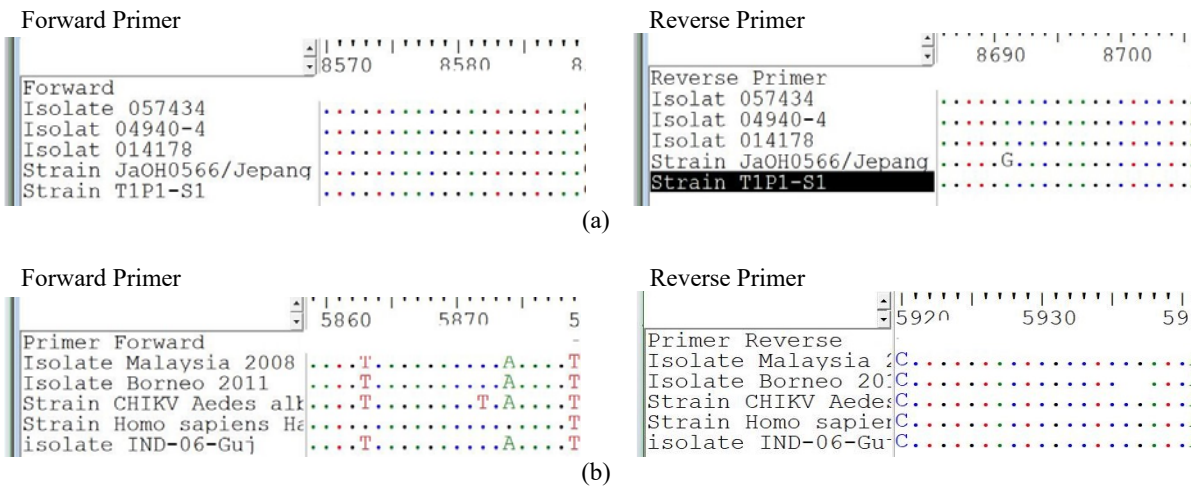Forward Primer   Reverse Primer

(b)

Figure 2. Alignment JEV primer (a) and CHIKV primer (b) with five different isolate

Table 4. Specificity test of JEV and CHIKV primer

Primers	RNA DENV-2		Healthy Person Plasma		Positive Control	
	Ct	Tm (°C)	Ct	Tm (°C)	Ct	Tm (°C)
JEV	N/A	-	N/A	-	18.93	83.18
CHIKV	34.57	83	N/A	-	18.88	85.5

N/A: negative result

Discussion

This study used two primer pairs for JEV and CHIK molecular detection with different gene target. Primer CHIKV used NSP4 gene as gene target and different with other study that used capsid gene and NSP1 as gene target. This study used NSP4 gene because it has conserved and specific region. Primer JEV in this study used NS5 as gene target different with other study that used NS3 as gene target. NS5 as non structural gene really important for RNA virus replication. NS5 gene act as methyltransferase, RNA guaniltransferase, and RNA-dependent RNA polymerase.[9] Conservation of primer sequence is showed by alignment of the primers at Figure 2. Besides the gene target, considering the primer condition is very important. The considerations are the size of primer with range 18-24 bp[10], GC content with range 40 to 70%[11], primer Tm between 59 °C and 68 °C with difference between forward and reverse around 1 to 2 °C[12,10] that has been fulfilled by JEV and CHIKV primer either forward or reverse primer. SYBR Green I detecting the amplicon by binding to non specific double stranded DNA, therefore it need to minimize the primer dimer to be occurred. Primer dimer usually occur because of self complementarity and 3' self complementarity between the primer.[13] JEV and CHIKV primers have minor of self complementarity and 3' self complementarity values. The primer in this study has fulfilled the primer condition requirement therefore it can be used for the RT PCR.

Annealing temperature for the primer is very important to be known for the reaction to work. The optimal primer annealing temperature could be determined by the best Ct and melting temperature analysis that was obtained from the gradient temperature reaction.[11] Virus in JE patient blood can be found in acute phase of the disease[14], in consequences real time RT PCR is useful at acute phase when the titer of viremia is high. Patient with JEV IgM positive, had not positive result in detection virus using RT PCR in plasma.[14] The other study in India had been testing RT PCR to patient that diagnosed JE with JEV IgM positive and the result was only 22.5% patient detected the JEV RNA.[15]

The detection limit from JEV primer in our study was at 10^{-5} pg concentration that equal to 4355 DNA copies at one reaction with Ct value 36.993. Bharucha et al use JEV primer with NS2A, NS5, and NS3 as gene target can detect 39 RNA copies and 4 RNA copies per one reaction.[16] Even though the detection limit in this study was higher, this detection limit still could detect the JE patient with viremia based on study before (Santhosh et al.). Santhosh et al. investigated acute encephalitis patient (1 to 7 days) in India and the average of JEV concentration in CSF patient was 20-2 x 10^3 RNA

copies and some patients had JEV concentration at 2×10^5 copy RNA per reaction.[17]

To test specificity, DENV-2 RNA and healthy person sample showed there was no cross reactivity between JEV and CHIKV primer with DENV-2 RNA. JEV primer produced negative result, but CHIKV primer resulted in amplification with Ct value at 37.048 and Tm at 79.24 °C, which was different from the amplicon from positive control. SYBR Green I is non specific reporter binding dyes and will detect any double stranded DNA such as primer dimer or artefact.[18] The specificity using healthy person plasma showed negative using those 2 primers. Our study only tests specificity against DENV, since it is endemic virus in the country, and the inavailability of another virus in our laboratory. Our in silico study showed that the primers did not bind to other viruses (ZIKV, YFV, WNV, and Hantavirus).

Conclusions

This study showed our inhouse primers optimal condition for real time RT PCR for detection of JEV and CHIKV, that can be developed further for diagnostic tools for patient with dengue-like fever symptoms.

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Conflict of Interest Statement

There were no coflicts of interest.

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