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Cloning of Chikungunya Virus E2 Gene in *Escherichia coli* TOP 10

B. S. P. Erlyandi[1], S. Pambudi[2], P. M. Tampubolon[1], F. A. Sitepu[1] and R. Lestari[1, a)]

[1]*Department of Biology, Faculty of Mathematics and Natural Sciences (FMIPA), Universitas Indonesia, Depok 16424, Indonesia*
[2]*Center for Pharmaceutical and Medical Technology, Badan Pengkajian dan Penerapan Teknologi (BPPT), Serpong 15310, Indonesia*

[a)]Corresponding author: retno.lestari.budiman@gmail.com

Abstract. Chikungunya virus (CHIKV) infection generally occurs in tropical and temperate regions such as Indonesia and causes a large amount of socio-economic loss. People infected with CHIKV experience severe arthralgia that can last for months to years. Nowadays, there are no low cost and powerful diagnostics system that can effectively prevent and diagnose CHIKV infection. For that reason, this study focuses on development of simple, robust, and rapid diagnostic assay for chikungunya virus infection. The aim of this study is creating recombinant plasmid vector with molecule size 7.223 bp. Here, the 1.260 bp of gene insert *envelope 2* (*E2*) protein CHIKV was cloned in 5.963 bp pYES2/CT vector. This recombinant vector transformed to *Escherichia coli* TOP 10 then plated in ampicillin selective agar medium. Several positive colonies of *E. coli* recombinant were isolated to obtain its plasmid molecule. Further step of confirmation recombinant vector analysis was done by digestion and PCR methods. The results of digestion method showed that two DNA bands appear with each size 5.963 bp and 1.260 bp while PCR method showed DNA bands by the size 1.260 bp and 1.560 bp. The conclusion is we have successfully cloned the CHIKV E2 protein in vector plasmid pYES2/CT based on digestion and PCR method.

Keywords*:* Antibody; cloning; dengue; heavy chain; PCR

INTRODUCTION

Chikungunya virus (CHIKV), classified into the *Togaviridae* family *Alphavirus* genus, is the virus that causes Chikungunya fever [1]. Symptoms of Chikungunya fever are very similar to dengue fever and Zika [2]. As a result, physical diagnosis of those infection is considered to be less accurate. Therefore, a method to ensure Chikungunya virus infection is crucial to do.

Several methods for detecting Chikungunya infections have been developed. Detection of Chikungunya RNA viral can be done in laboratories with real-time PCR [3]. This method can be used to detect CHIKV in patients' serum samples taken less than six days after the fever phase. Detection of CHIKV infection using real-time PCR has high sensitivity, but this method requires sophisticated facilities, trained human resources, considerable processing time, and high operational costs. These constraints caused the detection of CHIKV infection to be ineffective, both in cost and time, thus slowing the handling of patients [4].

Making fast, easy, inexpensive, and accurate detection kits are necessary to improve the detection of CHIKV infection. Serological detection that can recognize IgG and/or IgM specific antibodies to the Chikungunya virus can be an alternative as a detection system that is easy to use, inexpensive and also has a high sensitivity. The serological

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method can effectively detect Chikungunya infection in the patient's serum on day 5 after fever [5]. The development of a Chikungunya detection kit requires material in the form of a protein antigen derived from the Chikungunya virus. Previous research has found that envelope antigens from the Chikungunya virus can react with IgG and/or specific IgM antibodies in the serum of Chikungunya patients [2]. The development of recombinant envelope antigens in yeast cell expression systems can be an alternative in providing material for the development of Chikungunya detection kits [6].

Gene cloning is the initial stage in obtaining recombinant Chikungunya envelope antigen. The recombinant antigen is a product of expression of recombinant plasmids obtained through cloning envelope genes on pYES2/CT vectors. The recombinant plasmid was then cloned into *E. coli* cells to replicate and then transformed into *Saccharomyces cerevisiae* cells for protein expression. *E. coli* is used as the host cell to replicate the recombinant plasmid. Protein that is successfully expressed is still in the form of crude protein (crude extract) so a purification step needs to be done first. After successfully purifying and obtaining pure envelope protein, it can then be used in the development of detection kit products. The aim of thsi study is to create recombinant plasmid which the *E2* CHIKV gene is inserted in pYES2/CT plasmid vector. The successfully cloned *E2* CHIKV gene will go to protein expression and create the recombinant antigen.

MATERIALS AND METHOD

Phusion Hot Start II High–Fidelity DNA Polymerase and Phire HS II Master Mix for amplification of DNA target was obtained from Thermo-Fisher Scientific (Waltham, MA, USA). All used primers were obtained from Macrogen (USA). FastDigest *Hind*III and *Xba*I restriction enzyme was obtained from Thermo-Fisher Scientific (Waltham, MA, USA). The pYES2/CT vector was obtained from Thermo-Fisher Scientific (Waltham, MA, USA).

Sample of DNA Template

The DNA template of gene *E2* CHIKV was obtained from recombinant plasmid pET DEST-42-E2 CHIKV. Here, the gene *E2* CHIKV with molecule size 1260 bp was inserted in 7440 bp pET DEST-42 vector.

Polymerase Chain Reaction (PCR)

Polymerase Chain Reaction (PCR) reactions were performed both by using Phusion Hot Start II High–Fidelity DNA Polymerase [Invitrogen] and Phire HS II Master Mix [Invitrogen]. The sequences of primers for PCR reaction are shown in Table 1.

The reaction mixture was 20 µL containing pET DEST-42-E2 CHIKV as template, forward and reverse primers (10 µg/µL), dNTPs, polymerase enzyme, and Nuclease-Free Water up to 20 µL. The PCR reaction mixture and the PCR set-up are shown in Table 2. All reactions were carried out using a Bio-Rad PCR machine.

Separation of DNA fragments was done by agarose gel electrophoresis. It was performed using 0.8 % agarose gel [Invitrogen] and TAE running buffer in a gel electrophoresis apparatus [Takara] at 100 V for 30 minutes. The gel was stained by EtBr for visualization of the amplified DNA. Then, it was exposed to UV light for gel documentation.

TABLE 1. Sequences of *E2* CHIKV primers

Primer Name	Sequences
CHIKVE2pYES-Hind-Fw	5- TAATAAGCTTAAAAATGAGTATTAAGGACA ACTTCAATGTCTATAAAGCC -3
CHIKVE2pYES-Hind-Rv	5- TAATTCTAGACGCTTTAGCTGTTCTAATG CAGCATATTAGG -3
pYES2-Fw	5-AATATACCTCTATACTTTAACGTC-3
pYES2-Rv	5- GTCACGCTTACATTCACGC -3

TABLE 2. PCR reaction mixture and PCR set-up

Reagent	Final Concentration	Pre-Denaturation	Denaturation	Annealing	Final Extension
2x Phusion Green HS II HF Master Mix	1x	98 °C, 30 sec, 1 cycle	98 °C, 5 sec, 35 cycle	65 °C, 30 sec, 35 cycle	72 °C, 1 min, 1 cycle
Forward Primer	0.5 µL				
Reverse Primer	0.5 µL				
DNA Template	100 ng				
Nuclease-Free Water	-				

Cloning of *E2* CHIKV Gene into the Vector

The competent cells for transformation was done by cultivating the *E. coli* TOP10 in 3 mL of Luria-Bertani (LB) medium at 37 °C until OD_{600} is between 0.4–1. The cultures were transferred into 1.5 mL tube and cooled by storing on ice for 15 minutes. Furthermore, cell culture was centrifuged at 3000 rpm for 10 minutes at 4 °C. The supernatant was removed, then the formed pellets were resuspended with 50 mL of $CaCl_2$ 0.1 M cold. The cell culture was stored in ice for 30 minutes, then centrifuged at 4,000 rpm for 10 minutes at 4 °C. The pellets were resuspended with 400 µL of $CaCl_2$ 0.1 M.

Cloning of *E2* CHIKV into the vector was conducted by double digest method. Both sides of the gene contain two different restriction sites which was attached with the vector prior to ligation reaction, then the ligated products with molar ratio 1:5 of pYES2/CT vector and the DNA (insert) were used for transformation. Competent *E. coli* cells were transformed with ligated products using the heat shock method. Transformants were spread on LB agar plates with ampicillin (75 µg/mL) and the plates were incubated at 37 °C overnight.

Isolation of Recombinant Plasmid

The plasmid DNA was isolated from 10 colonies using alkaline lysis procedure [7]. *E. coli* TOP10 cells containing the recombinant plasmids were grown overnight at 37 °C in 3 mL of LB medium, 250 rpm in a shaker incubator. The cells were harvested in Eppendorf tube by centrifugation at 11,000 rpm for 30 seconds, the supernatant was then discarded. Then, 100 µL of Solution 1 (50 mM glucose, 25 mM Tris-HCl with pH 8.0, 10 mM Na_2EDTA, 100 µg/mL RNAse A) was added into the tube and the mixture was mixed by pipetting. After that, 200 µL of Solution 2 (0.2 M NaOH and 1 % SDS) was added and mixed by inversion 6–8 times. Next, 150 µL of Solution 3 (3 M NaOAc (sodium acetate) with pH 4.8) was added and mixed by inversion 6–8 times. The mixture was centrifuged at 13,000 rpm for 10 minutes at 4 °C, then the supernatant was removed. 1 mL of cold 100 % EtOH was added, then mixed by inversion and incubated at -30 °C for 1 hour. The tube was then centrifuged at 13,000 rpm for 10 minutes at 4 °C, and the supernatant was discarded. The pellets were washed with 500 µL cold 70 % EtOH, then was centrifuged at 13,000 rpm for 5 minutes at 4 °C and dried at room temperature. Finally, the pellets were dissolved in 50 µL Nuclease-Free Water and 1 µL was tested on agarose gel.

Verification of Cloning Result

The cloned *E2* CHIKV was verified by two methods; double digestion and PCR. The double digestion reaction with FastDigest *Hind*III and *Xba*I restriction enzyme consisted of 500–800 ng isolated recombinant plasmid DNA, 1 *Hind*III enzyme unit, 1 *Xba*I enzyme unit, 1x FD buffer, then the reaction mixture was incubated at 37 ° C for 2 hours. Digestion results were then analyzed by electrophoresis using DNA markers 1 kb PCRBIO Ladder I. Recombinant plasmids that show positive results at the verification with digestion are subsequently verified by PCR. The PCR reaction consisted of 1 µL recombinant plasmid, 1 µL of forward primer (0.5 µM) (5-AATATACCTCTATACTTTAACGTC-3) and 1 µL of reverse primer (0.5 µM) (5-GTCACGCTTACATTCACGC-3), mixed in 10 µL of 2x Phire HS II Master Mix and 7 Nuclease-Free Water. The condition of the PCR began with

initial denaturation at 98 °C for 30 seconds. The PCR cycle was 35 times starting with denaturation at 98 °C for 5 seconds, annealing at 60 °C for 30 seconds. The PCR cycle ended with final extension at 72 °C for 1 minutes and the incubation temperature decreased to 12 °C. The PCR products obtained were analyzed by 0.8 % agarose gel electrophoresis (100 V, 30 minutes).

RESULTS AND DISCUSSION

Amplification of *E2* CHIKV gene gives positive result by showing a DNA band with the size 1260 bp shown in Fig. 1. The molecule size of *E2* CHIKV gene is 1260 bp [8]. This means that the required gene for cloning had been successfully amplified. The gene was then inserted in vector pYES2/CT to create recombinant plasmid and transformed in *E. coli* TOP 10. The recombinant plasmid was isolated from ten colonies of *E. coli* TOP 10 and the OD 260/280 is measured to evaluate the quality. The results are shown in Table 3. Based on the results, it can be seen that the *E. coli* recombinant plasmid from 10 colonies have a good concentration and purity. According to [9], pure DNA has an OD260/OD280 ratio of 1.8–2.

Double digestion was done by using *Hind*III and *Xba*I restriction enzyme for verification of resulting clones. *Hind*III and *Xba*I restriction sites are found in MCS of pYES2/CT plasmid at 480th and 540th bases. After digestion, the positive clones were verified by PCR to evaluate *E2* CHIKV gene orientation using pYES primers. The digestion method result is shown in Fig. 1. The assumption is, if the *E2* CHIKV gene was successfully inserted on pYES2/CT vector (each 5 'end and 3' both DNA fragments complement each other), a band sized 5963 bp and 1260 bp will be shown on agarose gel electrophoresis. Furthermore, if the PCR product of *E2* CHIKV showed DNA bands by the size 1260 bp and 1580 bp, it means that the inserted gene had been integrated in vector with correct orientation. The results of recombinant plasmid verification by PCR method is shown in Fig. 2.

The DNA bands which appear on gel agarose electrophoresis of digestion method should only be two bands specific on size 5963 bp and 1260 bp. But in the results a third band appears with the additional band with a size of ~3000 bp. This result suggests that the digestion process had not been completed yet or in other words, a partial digestion. This occurs when there is too much recombinant plasmid sample while the amount of restriction enzyme is too low. So, it is necessary to have a longer duration of digestion for a complete plasmid digestion. Good DNA bands results, can be obtained by decreasing the amount of recombinant plasmid sample or increasing time duration of digestion process [10].

The confirmation of recombinant plasmid by PCR method is positive since DNA bands appear on gel agarose electrophoresis by the size 1260 bp and 1580 bp. The result can be seen in Fig. 2. Lane 1 is pYES2/CT amplified by PCR reaction using pYES primer. The band appears on size 320 bp because it only amplifying the MCS region. Lane 2 is the PCR reaction which amplifies *E2* CHIKV gene with pYES2/CT as DNA template. No band appears as no *E2* CHIKV gene is inserted in this plasmid. Lane 3 is positive control of *E2* CHIKV gene. Lane 4 is the sample of recombinant plasmid amplified with pYES primer. A eDNA band appeared with a 1580 bp size which comes from the total of inserted gene with a 1260 bp size plus MCS with a 320 bp size. Lane 5 is the sample of recombinant plasmid amplified with *E2* primer which showed band at 1260 bp.

TABLE 3. Concentration and OD260/280 DNA

Nucleic Acid	Concentration ng/µL	OD260/280
Colony 1	1923.2	1.891
Colony 2	2271.3	1.918
Colony 3	1522.3	1.883
Colony 4	1374.5	1.874
Colony 5	2072.3	1.892
Colony 6	2172.0	1.889
Colony 7	2637.8	1.938
Colony 8	11895	1.834
Colony 9	2058.4	1.903
Colony 10	1727.9	1.864

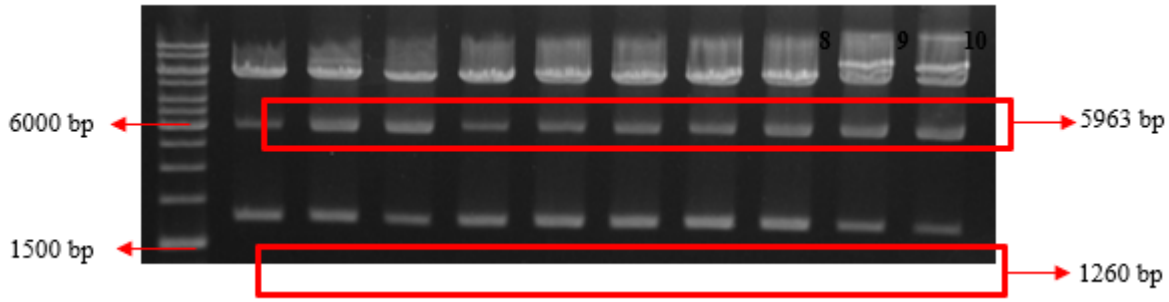

FIGURE 1. Verification of resulting clones. Double digestion of recombinant plasmids with *Hind*III and *Xba*I. M is ladder; lane 1-10 are *E. coli* colonies 1-10.

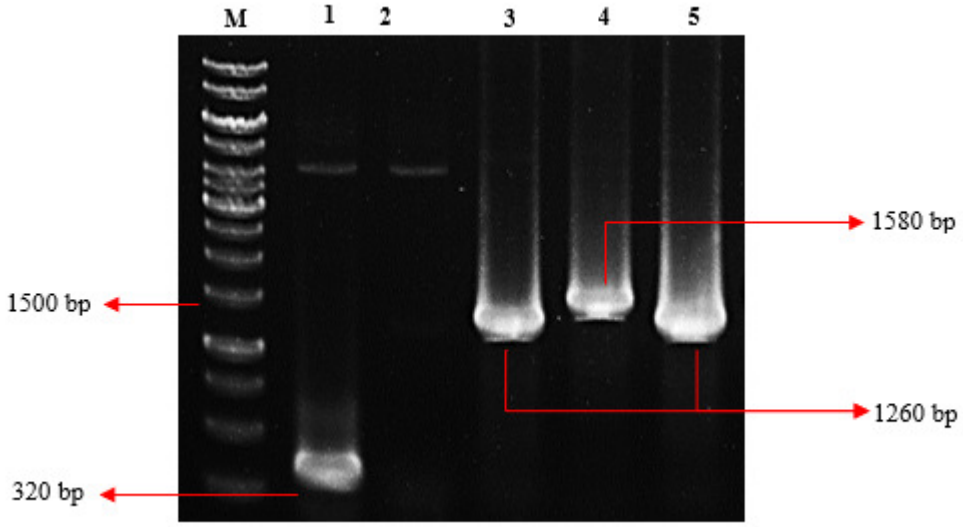

FIGURE 2. Verification of resulting clones. PCR of recombinant plasmids using *E2* CHIKV primers and pYES primers. M is ladder; lane 1 is pYES2/CT + primer pYES; lane 2 is pYES2/CT + primer *E2* CHIKV; lane 3 is positive control of *E2* CHIKV gene, lane 4 is recombinant plasmid + primer pYES; lane 5 is recombinant plasmid + primer *E2* CHIKV.

CONCLUSION

The results showed that the *E2* CHIKV gene sized 1260 bp was successfully cloned in pYES2/CT vector inside *E. coli* TOP 10 and confirmed by digestion and PCR methods.

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REFERENCES

1. D. Diop, G. Meseznikov and M Sanicas, MOJ Public Health **2**, 181-187 (2015).
2. B. Cho et al., Yonsei Med. J. **49**, 828-835 (2008).
3. P. Grivard et al., Pathol. Biol. **55**, 490-494 (2007).
4. E. Burdino, G. Calleri, P. Caramello and V. Ghisetti, Emerg. Infect. Dis. **22**, 1837-1839 (2016).
5. B. W. Johnson, B. J. Russell and C. H. Goodman, J. Infect. Dis. **214**, S471-S474 (2016).
6. S. Saraswat, T. N. Athmaram, M. Parida, M. Agarwal, A. Saha and P. K. Dash, PLoS Neglec. Trop. D. **10**, (2016).
7. H. C. Bimboim and J. Doly, Nucleic Acids Res. **7**, 1513-1523 (1979).
8. A. K. Verma, A. Chandele, M. Kaja, A. Arulandu and P. Ray, BMC Infect. Dis. **14**, P65 (2014)
9. C. F. Barbas III, D. R. Burton, J. K. Scott and G. J. Silverman, C. S. H. Protoc., (2007) available at: http://cshprotocols.cshlp.org/content/2007/11/pdb.ip47.long
10. P. N. Hengen, Trends Biochem. Sci. **21**, 441-442 (1996).