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Cloning of Chikungunya Virus Envelope 1 (E1) Gene to pYES2/CT in *Escherichia coli* TOP10

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Abstract. Chikungunya virus infection (CHIKV) causes chikungunya disease to humans which is transmitted by the *Aedes* mosquito. The disease causes acute fever, arthritis, and arthralgia. Chikungunya infection has been endemic in Indonesia, especially in Jambi. Detection of chikungunya disease which still uses the PCR method requires high costs. This reasoning is the basis of our research to develop a simple, fast, and accurate diagnostic test for chikungunya disease. A 7,283 bp recombinant vector carrying the CHIKV E1 (Envelope 1) gene was developed. The E1 gene measuring 1,320 bp was cloned in a pYES2/CT vector measuring 5,963 bp. The recombinant vector was then transformed into *Escherichia coli* TOP10 using the Transformation and Storage Solution (TSS). Transformant selection was cultured in agar medium with ampicillin antibiotics and colonies were isolated for recombinant plasmid extraction. Confirmation of the E1 gene existence was done using pYES2/CT with digestion and PCR. The results of pYES2/CT digestion electrophoresis obtained two DNA bands with a size of 1,320 and 5,963 bp, whereas the PCR method was obtained 1,640 bp and 1,320 bp. These results indicate the process of cloning the E1 CHIKV gene to pYES2/CT was successful based on confirmation with the digestion and PCR methods.

Keywords*:* Chikungunya, E1, *Escherichia coli* TOP10, pYES2/CT

INTRODUCTION

Chikungunya fever (CHIK) is an arbovirus disease caused by the chikungunya virus (CHIKV) passing transmitted by the Aedes mosquito. The chikungunya virus belongs to the Togaviridae family with the genus Alphavirus, has an envelope and positive single stranded (ss) RNA material [1]. The Chikungunya case in Indonesia was first recorded in 1972 but spread again in 2001 [2]. There were 137,655 Chikungunya cases that occurred in Indonesia in 2009-2010. Chikungunya in Indonesia is classified as mostly from the Asian genotype virus and few ECSA genotypes (East, Central, South Africa) [3].

Symptoms of CHIK disease are difficult to distinguish accurately from dengue disease. Common symptoms of CHIK disease such as high fever, joint and muscle pain, rash, and headache. Cases of CHIK symptoms in adults cause arthralgia, alopecia, and depression. An analysis showing the effects of CHIK symptoms can reduce the economic productivity of the community [3]. Currently there is no licensed vaccine or antiviral therapy for CHIK disease [4]. The development of a diagnosis of CHIK is very important in the rapid management of these symptoms as well as symptoms similar to those of DHF [5]. Current diagnosis of CHIK is developing such as detection by RT-PCR, ELISA, immunochromatographic rapid tests [6–8].

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The CHIKV genome is about 12 kb in size consisting of a gene encoding a nonstructural protein (nsP1-4) and a structural protein coding gene (C, E1-3, and 6 K). Chikungunya virus genotypes based on genetic analysis identified three genotypes, West African, East/Central/South African (ECSA), and Asian. One of the CHIKV structural proteins, Envelope 1 (E1) has been studied for the development of CHIK detection. Epitopes of these proteins are more reactive than E2 proteins [9]. Yathi et al. [10] showed recombinant E1 protein as a high immunospecific protein and a good target for serodiagnosis.

Amplification of the E1 CHIKV gene for protein production can be done on prokaryotic cells. The E1 CHIKV gene was obtained from Jambi which is an Asian genotype [11]. Research on the cloning of the E1 CHIKV gene in *Escherichia coli* using pYES2/CT aims for the protein to be expressed from eukaryotic cells (yeast). Previous research used hosts for prokaryotic or baculovirus expressions [10–13]. The E1 gene that has been propagated using a prokaryotic cell system and expressed with eukaryotic cells produces recombinant E1 proteins that are not toxic and do not form inclusion bodies. In the future, these proteins can be produced for the ingredients of making early diagnosis of CHIK.

MATERIALS AND METHOD

Envelope 1 CHIKV Gene

The E1 CHIKV gene was obtained from the Eijkman Institute which was inserted in pET-21. Envelope 1 Gene was amplified from pET-21-E1 using primer which can be seen in Table 1. The gene is 1,320 bp. Amplification was carried out with a three step protocol beginning with the first cycle of the initial denaturation of 98 °C for 30 seconds; the second cycle with a denaturation of 98 °C for 30 seconds, annealing at 65 °C for 30 seconds, and extension at 72 °C for 15 seconds with repetition of the second cycle 30 times; The third cycle begins with the final extension at 72 °C for 5 minutes and ends at 12 °C. Visualization of the amplification results by electrophoresis on agarose gel 1 % and running at 200 volts for 20 minutes. The PCR results were purified as insert genes for cloning with the Wizard® SV Gel and PCR Clean-Up System Kit [Promega].

Cloning to *E. coli* TOP10

Gen E1 purification results were inserted into pYES2/CT [ThermoFisher]. The competent cell used was *E. coli* TOP10 [Invitrogen] and the method was from Chung et al. [14]. Plasmid and E1 genes that have been digested with HindIII and XhoI were ligated with Anza™ T4 DNA Ligase Master Mix [ThermoFisher] with plasmid E1 genes ratio 1:9 and 1:4. Transformation was mediated with PEG based on the method from Chung et al. [14]. The transformation results were cultured with LB agar + Ampicillin [Sigma Aldrich] (100 µg/mL) medium which was distributed into negative controls (*E. coli* without transformation), positive control (pYES2/CT *E. coli* transformation), and two mediums with transformation results from vector comparison: inserts 1:9 and 1:4.

Cloning Verification

Colonies that grew on the medium were isolated into LB agar + Ampicillin (100 µg/mL). Wizard® Plus SV Minipreps DNA Purification System Kit [Promega] was used. Cell pellets which have been grown overnight were separated by centrifugation. Cell Resuspension Solution, Cell Lysis Solution, Alkaline Protease Solution, and Neutralization Solution were added, and the mixture was centrifuged. The mixture was put into the spin column and the lysate was decanted. Centrifugation was continued and the previous steps were repeated. The spin column was moved to a new tube and after addition of nuclease-free water centrifugation was continued Cloning verification was carried out by double digestion with HindIII and XhoI restriction enzymes as well as pYES2/CT multiple cloning site amplification and E1 gene [15]. The primers used for pYES2/CT and E1 gene amplification can be seen in Table 1. The verification results are visualized with 1 % agarose gel and running at 200 volts for 20 minutes.

RESULTS AND DISCUSSION

The E1 gene was successfully amplified with primers attached to the HindIII and XhoI restriction enzyme sites. The E1 gene amplification was obtained by the band which was estimated at the size of 1,320 bp shown in Fig. 1. The annealing temperature of 65 °C was obtained by the visible band and the smear was below the marker. The smear was formed from a primer that has dimers. The amplification results were purified for the digestion process.

The pYES2/CT digestion and E1 gene used HindIII and XhoI restriction enzymes at 37 °C for three hours. The pYES2/CT ligation and E1 gene were carried out in vitro to the competent *E. coli* TOP10 cell in an antibiotic-added medium. The transformation results obtained bacterial colonies on positive and medium control medium with a ligation ratio of 1:9 (Fig. 2b). Transformant colonies can grow on ampicillin medium because they have plasmids with ampicillin resistant selective markers [16]. Five transformant colonies with ligation ratio of 1:9 and pYES2/CT transformant colonies will then be isolated by plasmids. The transformation efficiency obtained is 0.5 cfu/µg plasmid. This value is less than the results of the transformation efficiency of the transformation method Chung et al. [14]. The use of TSS solution contains PEG which does not affect the efficiency of the transformation [17].

TABLE 1. Sequences of CHIKV E1 and pYES2/CT primers.

Primer Name	Sequences (5' to 3')
CHIKVE1-HindIII-Fw	TAATAAGCTTAAAAATGTACGAACACGTAACAGTGATCCCGAACACG
CHIKVE1-XhoI-Rv	TAATCTCGAGGTGCCTGCTAAACGACACGCATAGCACC
pYES2-Fw	AATATACCTCTATACTTTAACGTC
pYES2-Rv	GCGTGAATGTAAGCGTGAC

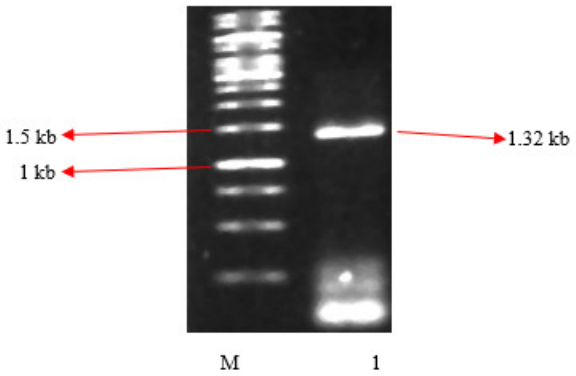

FIGURE 1. Agarose gel 1 % electrophoresis (200 v, 20 min) of E1 gene amplification. M: DNA Ladder 1 kb, 1: E1 Gene.

(a) (b)

FIGURE 2. Transformant colonies. Images with circled red indicate the growth of colonies.
(a) negative control, (b) ligation vector: insert = 1:9.

The isolation of plasmids from transformant colonies was followed by spectrophotometry analysis to determine DNA purity and concentration. The purity level (A_{260}/A_{280}) from the plasmid isolation is in the range of 1.8, then the recombinant plasmid concentrations are in the range 324.3–434.9 ng/µl. The results can be seen in Table 2. Plasmid isolation that were visualized by electrophoresis obtained three and five colonies thought to carry recombinant plasmids. Electrophoresis results can be seen in Fig. 3.

Verification of recombinant plasmids by double digestion using HindIII and XhoI restriction enzymes. The process was carried out at 37 °C for three hours. Visualization of the double digestion results by electrophoresis obtained three and five colonies with two bands. The two bands are expected to be in the size of 5.9 kbp and 1.33 kbp. It is suspected that the two bands were pYES2/CT and the E1 CHIKV gene. The results of visualization can be seen in Fig. 4.

TABLE 2. Concentration and A_{260}/A_{280} of isolated plasmid DNA.

Clone	Concentration (ng/µl)	A_{260}/A_{280}
I	324.3	1.77
II	421.1	1.83
III	398.4	1.87
IV	414.5	1.79
V	334.9	1.85

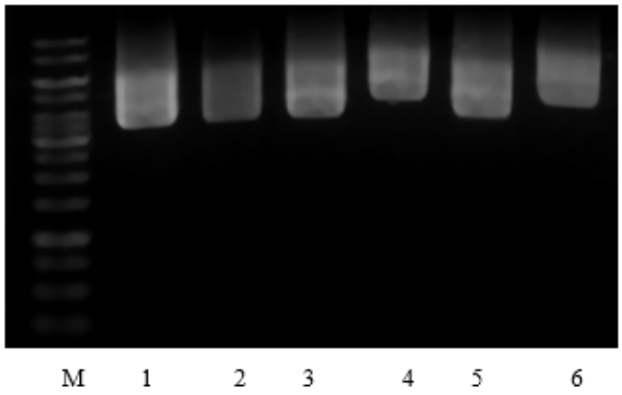

FIGURE 3. Agarose gel 1 % electrophoresis (200 v, 20 min) of isolation recombinant plasmid. M: DNA ladder 1 kb, 1: first colony, 2: second colony, 3: third colony, 4: fourth colony, 5: fifth colony.

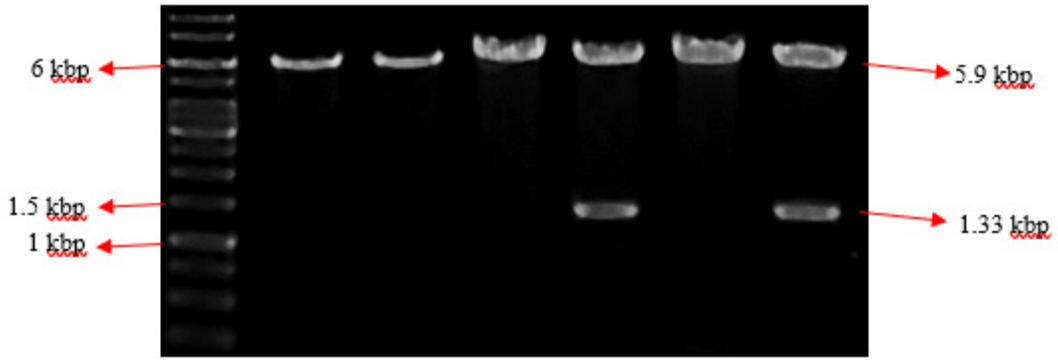

FIGURE 4. Agarose gel 1 % electrophoresis (200 v, 20 min) of double digest using HindIII and XhoI. The 3rd and 5th colonies were thought to carry the plasmid insert. M: DNA ladder 1 kb, 1: first colony, 2: second colony, 3: third colony, 4: fourth colony, 5: fifth colony.

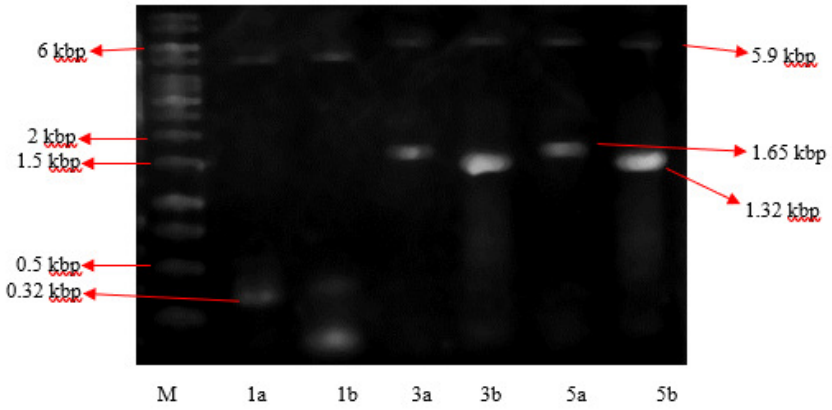

FIGURE 5. Agarose gel 1 % electrophoresis (200 v, 20 min) of verification recombinant plasmid using PCR. M: DNA ladder 1 kb, a: amplification with pYES2/CT primers, b: amplification with E1 primers, 1: first colony, 3: third colony, 5: fifth colony.

Recombinant plasmid verification was then performed by PCR using primary E1 gene and pYES2/CT primer. Amplification was carried out on three and five colonies suspected of carrying recombinant plasmids. The visualization of electrophoresis obtained a band that was estimated to be 1,345 bp size with E1 primary amplification can be seen in Fig. 5. The pYES2/CT primers attached to multiple cloning sites (MCS) with a product size of 331 bp. Amplicon with pYES2/CT primer formed a band at the size of 1,620 bp. Recombinant plasmid verification by PCR showed the third and fifth colonies carrying recombinant pYES2-E1 plasmid.

The third and fifth colonies with double digestion and amplification verification show that there are two bands, one of which is the E1 gene band. Based on the results of plasmid isolation, the third and fifth colonies are higher than other colony band sizes. Amplification with multiple cloning site primers was used to determine the E1 gene cloned at that site in pYES2/CT. Multiple cloning sites have promoter sequences, V5-tags, and His-tags function for protein expression, immunoblotting, and protein purification [18]. Recombinant plasmid verification will be followed by DNA sequencing to determine the success of protein expression.

CONCLUSION

Cloning the E1 gene to pYES2/CT on *E. coli* TOP10 was successfully carried out by PEG transformation. In the future, cloning results can be sequenced and continued to be expressed in yeast (*Saccaromyces cerevisiae*).

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