J Med Biosci Suppl 2025;7: S29 - S33 DOI: 10.58837/CHULA.JMBS.7.S1.5

Original article

Development of chikungunya virus detection by CRISPR-Cas12a-based detection

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Abstract

***Background**:* The chikungunya virus is a mosquito-borne virus that causes acute fever with potentially prolonged joint pain. Aedes aegypti and Aedes albopictus are primary vectors for chikungunya in Asia, as well as dengue and Zika viruses. The viruses co-circulate in the same habitat and cause a similar acute fever in early onset, posing a misdiagnosis challenge. The reverse transcription-polymerase chain reaction is the only diagnostic option for detecting the chikungunya viral gene.

***Objective**:* This study aimed to evaluate the designed primers and crRNAs for a molecular assay of chikungunya viral gene detection using RT-RPA amplification coupled with a CRISPR-Cas12a detection system.

***Methods**:* Chikungunya virus complete genome sequences available from National center for biotechnology information (NCBI) were aligned using Jalview to identify potential crRNAs and primers in conserved regions. The designed recombinase polymerase amplification (RPA) primers and crRNAs were validated to determine the optimal primers and crRNAs.

***Results**:* Following the alignment of Chikungunya viral genome sequences, primers and crRNAs were designed for the nsP2 gene, which is a conserved region suitable for detection. The designed primers and crRNAs were successfully used in CHIK gene amplification and detection.

***Conclusion**:* This study offers a molecular assay that could be a potential diagnostic test for chikungunya viral detection. Further investigations include evaluation for the limit of detection, cross-reactivity, and clinical sensitivity and specificity tests.

***Keywords**:* Chikungunya, CRISPR, detection, RPA.

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The mosquito-borne diseases have been an alarming concern with global climate change and urbanization.[1] Chikungunya virus is an arbovirus transmitted by mosquitoes and is usually incubated for 3 to 7 days after an infected mosquito bite. The most common symptoms are fever and joint pain; other symptoms may include headache, muscle pain, joint swelling, or rash.[2] Although the symptoms of chikungunya virus infection are usually mild, certain patients of extreme ages could develop persistent chronic arthritis or neurological complications (World Health).[3] Due to their clinical manifestations being similar to those of other arboviral infections, particularly dengue fever, people infected with the chikungunya virus are either underdiagnosed or misdiagnosed.[4] Therefore, an accurate early diagnosis of acute chikungunya infection is essential to determine the most appropriate treatment.

Chikungunya virus (CHIKV) is a positive-sense, single-stranded RNA virus from the Togaviridae family and Alphavirus genus, with an ~11.8 kb genome comprising two main open reading frames (ORFs).[5] The 5' ORF encodes non-structural proteins (nsP1–nsP4) essential for RNA replication, while the 3' ORF encodes structural proteins (C, E3, E2, 6K, E1) required for virion assembly. CHIKV enters host cells via clathrin-mediated endocytosis.[6] The viral RNA is released into the cytoplasm and immediately translated to produce the nsP1–nsP4 polyprotein. The viral RNA-dependent RNA polymerase (nsP4) synthesizes a negative-sense RNA intermediate, serving as a template for generating new genomic RNA and a subgenomic RNA (26S mRNA) for structural protein synthesis. The subgenomic RNA is translated into structural proteins (C, E3, E2, 6K, E1) in the endoplasmic reticulum (ER). These proteins are processed in the ER-Golgi and transported to the plasma membrane for assembly. The nucleocapsid assembles with genomic RNA, and the virus buds from the membrane, acquiring its lipid envelope.[7] CHIKV is identified in 3 genotypes: Asian, East-Central-South-African (ECSA), and West African (WA).[8] The CHIKV outbreak across the Indian Ocean, which spread from Kenya in 2004, caused mutations to the original virus and was categorized as a sub-lineage of ECSA known as the Indian Ocean Lineage (IOL).[5]

Currently, molecular detection by polymerase chain reaction (PCR) is the only method available to diagnose CHIKV acute infection.[9] However, the PCR technique requires a thermal cycler, which can be challenging to implement in vulnerable areas with limited access to healthcare resources.[10] Thus, an uncomplicated, rapid, cost-effective, and specific molecular assay could significantly enhance the diagnosis of chikungunya infection.

The isothermal amplification technique (IAT) of nucleic acids is an amplification of nucleic acid at a steady temperature and without requiring thermocycling, which can be performed in a simple laboratory.[11] Among IATs, recombinase polymerase amplification (RPA) is known for its outstanding sensitivity, specificity, rapid amplification, and simplicity.[12] The RPA technology has been established to diagnose Ebola virus and dengue virus in field settings.[13, 14]

The clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein (Cas) system refers to a sensitive biological system that identifies foreign genes and cleaves them by RNA-guides called CRISPR RNA (crRNA) to protect its host.[15] Based on the established requirement for Cas protein activation, CRISPR–Cas systems have been developed for various applications, including the detection of infectious diseases.[16] CRISPR-Cas12a is introduced in molecular diagnostic applications such as RPA to enhance sensitivity, specificity, and cost-effectiveness.[17]

CRISPR-Cas12a has collateral strand cleavage activity, non-specifically cleaving both double-stranded and single-stranded DNA with the help of crRNA after encountering the target sequence. With this characteristic, a single-stranded DNA reporter molecule is designed to detect fluorescent signals once Cas12a recognizes its target.[18] The PAM sequence of Cas12a is TTTV, and V can be A, C, or G. The crRNA of Cas12a is approximately 40-44 bases long and contains a direct repeat (DR) and spacer.[19]

Therefore, this study aims to evaluate the designed primers and crRNAs for a molecular assay of chikungunya viral gene detection using RT-RPA amplification coupled with a CRISPR-Cas12a detection system.

Materials and methods

Primer and crRNA design

An alignment of Chikungunya virus complete genome sequences available from National center for biotechnology information (NCBI) (https://www.ncbi.nlm.nih.gov/nuccore) that cover all three genotypes plus sub-lineage was performed using Jalview to identify potential crRNAs and primers in the conserved regions.

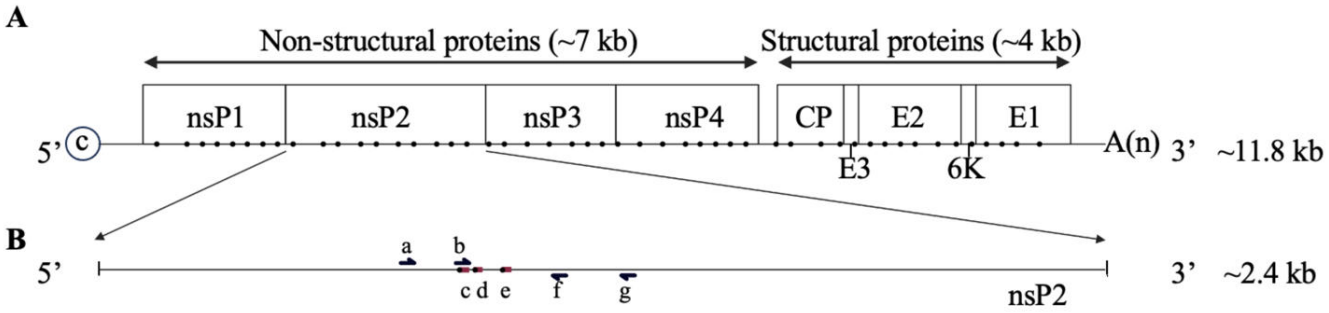

Figure 1. Schematic of the CHIKV genome sequence. **(A)** Black dots represent PAM (5' TTTV) on CHIKV genome sequence which V can be A, C or G. **(B)** Designed crRNAs and primers on nsP2 gene, where (a) and (b) represent forward primers; (c), (d) and (e) represent crRNAs; (f) and (g) represent reverse primers.

crRNA was designed based on the following requirements: a short nucleic acid sequence called 5' TTTV (where V can be A, C, or G) protospacer adjacent motif (PAM) is located next to the target site. crRNA is a 20-nucleotide sequence that must complement the target site (**Figure 1A**).

Consequently, primers were designed to cover crRNA spacer sequences and were verified for properties using the web-based tool PRIMER BLAST (https://www.ncbi.nlm.nih. gov/tools /primer-blast/). The designed crRNAs and primers were on the conserved region of the CHIKV nsP2 gene (**Figure 1B**).

Preparation for crRNA

The crRNA was designed in oligonucleotides with a direct repeat and T7 promoter as a template. The crRNA template was annealed with the T7 promoter with a final concentration of 4 µM in 1X T4 DNA Ligase buffer. The reaction was incubated in the following conditions: 95 °C for 3 minutes, 65 °C for 3 minutes, 42 °C for 5 minutes, and 37 °C for 45 minutes. The product from annealing was quantified by a nanodrop spectrophotometer.

The annealed template was used in an *in vitro* transcription to generate crRNA using Riboprobe In Vitro Transcription (IVT) T7 RNA polymerase System (Promega, USA) according to the manufacturer's protocol. Then 2µl of RQ1 RNase-Free DNase was added to each tube, followed by incubation at 37 °C for 10 minutes. After that, the transcribed RNA was treated with 5 µl of enzyme DNase I (Sigma-Aldrich, USA) and 5 µl of 10x Reaction buffer (Sigma-Aldrich, USA) at room temperature for 5 hours as an extra step for DNA template removal.

The IVT product (transcribed crRNA, approximately 40 nucleotides) was purified by miRNA isolation kit (Geneaid, Taiwan) and quantified by Qubit™ microRNA Assay Kit (Thermo Scientific™, USA).

Genomic RNA extraction

RNA extraction of CHIKV (lab strain) from infected cells was performed according to the manufacturer's protocol as described in the ZYMO Research Quick-RNA Viral Kit.

RT-RPA

RT-RPA assay was performed using the TwistAmp® Basic kit. Each reaction consists of 0.48 µM forward primer, 0.48 µM reverse primer, 40 U RevertAid Reverse Transcriptase (Thermo ScientificTM, USA), and rehydration buffer that is mixed with the lyophilized reaction of the TwistAmp® Basic kit (TwistAmp®, UK). After that, add 1 µl of RNA template and 14 mM of MgOAc. Then, incubate at 39 °C for 30 minutes, followed by 75 °C for 5 minutes to deactivate the reaction.

CRISPR-Cas12a

The reaction of CRISPR-Cas12a-based nucleic acid detection was prepared in a PCR microtube. Each reaction consisted of 30 nM of crRNA, 50 nM of EnGen® Lba Cas12a (Cpf1) (New England Biolabs, USA), 1X NEBuffer 2.0 reaction buffer (New England Biolabs, USA), 200 nM of FQ reporter, and 1µl of RPA product. The total volume was adjusted to 15 µl with nuclease-free water. Then, it was incubated at 39 °C for 15 minutes, and the BluPAD Dual LED Blue/White Light Transilluminator was used to visualize the fluorescent signal.

Results

RT-RPA amplification

To test the performance of RPA primers as shown in **Table 1**, two forward primers and two reverse primers were used to amplify CHIKV viral RNA (**Figure 1**).

Subsequently, the primer pair CH2320F-CH2693R (**Figures 2 and 3**) was selected for further experiments due to the amplified gene. The results from gel electrophoresis (**Figure 3**) indicated that all primer pairs successfully amplified CHIKV. we found higher intensity compared to the products amplified by the other primer pairs (**Figure 3**).

crRNAs evaluation by CRISPR-Cas12a detection of CHIKV

To evaluate the performance of CRISPR-Cas12a detection of CHIKV, the designed crRNA1, crRNA2, and crRNA3 were utilized in the CRISPR-Cas12a detection system. The performance of crRNAs with the RPA-amplified product of CHIKV was evaluated through fluorescence emission using a blue light transilluminator, which was observable to the naked eye. As anticipated, all reactions showed strong fluorescence emission (**Figure 4**). crRNA1 was chosen for further study due to its sequence having no mismatches with all CHIKV genotypes (data not shown).

Discussion

Chikungunya virus is a mosquito-borne viral infection that is often underestimated due to its symptoms are similar to those of other viral infections, such as Dengue and Zika. Therefore, early diagnosis is necessary to select the best treatment approach. The gold standard of acute CHIKV detection is molecular assay by PCR, which is challenging to perform in low healthcare system areas.[10] Therefore, we have developed a molecular diagnostic test by RPA coupled with CRISPR-Cas12a detection of CHIKV.

After a complete CHIKV genome alignment, the primers and crRNAs were designed for the conserved region of the nsP2 gene. The RT-RPA reaction with the designed primers successfully amplified the CHIKV gene. To increase the specificity of the assay, we combined the RPA amplified product with the CRISPR-Cas12a reaction.

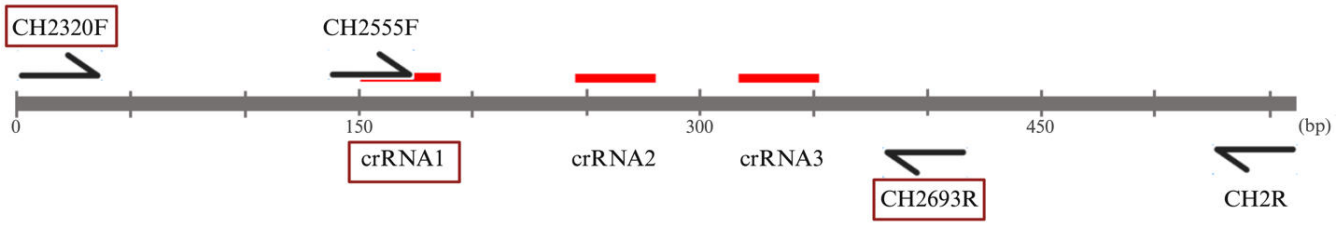

Figure 2. Schematic of primers and crRNAs on the nsP2 gene. Red boxes indicate selected primers and crRNA.

Table 1. Primers and oligonucleotides used in this study.

Name	Sequence (5'-3')
CH2320F[a]	TTGAATGGATGCAACAGACCAGTCGACGTGTT
CH2555F	TGCGGCTTCTTCAATATGATGCAGATGAAAGT
CH2693R[a]	TCCACGATAGTCAATTTGCAGTTGTTTAACCC
CH2R	GCGGCTTCTTCAATATGATGCAGATGAAAGTC
T7_CH2320F	TAATACGACTCACTATAGGGTTGAATGGATGCAACAGAC CAGTCGACGTGTT
T7 promoter	TAATACGACTCACTATAGGG
crRNA1	TTCTTCAATATGATGCAGATATCTACACTTAGTAGAAATT ACCCTATAGTGAGTCGTATTA
crRNA2	ATCTGCACCCAAGTGTACCAATCTACACTTAGTAGAAA TTACCCTATAGTGAGTCGTATTA
crRNA3	TCATCGTTGCATTACGAAGGATCTACACTTAGTAGAAA TTACCCTATAGTGAGTCGTATTA
ssDNA-FQ[b]	FAM-AGGACCCGTATTCCCA-BQH1

Note: spacer is underlined. [a]Selected RPA primers. [b]Trans cleavage reporter for Cas12a

CRISPR-Cas12a has collateral strand cleavage activity, cleaving non-specifically. It can cleave double-stranded DNA as well as single-strand DNA with the help of crRNA after binding to the target sequence that is complementary to the crRNA. With this characteristic, the Cas protein can be designed to bind and cleave the target nucleotides by including the proper crRNA and a single-stranded DNA reporter molecule to detect fluorescent signals when Cas12a recognizes its target.[18] The results demonstrate that the designed crRNAs could be effectively employed in the Cas12a reaction assay to detect the amplified gene.

Misiurina, et al. reported that a single nucleotide mismatch of crRNA could significantly decrease Cas12a catalytic activity due to a lack of complementarity between crRNA and the target site.[20] Previous studies reported the use of CRISPR-Cas12a for CHIKV detection. However, the crRNAs used in those studies contained nucleotide mismatches that were not complementary to all CHIKV genotypes, which could reduce or eliminate their ability to detect specific genotypes. [21, 22] Therefore, crRNA1 was selected for additional investigation because its sequence matched perfectly with the target site of all CHIKV genotypes.

However, several limitations were considered in this study. First, cross-reactivity tests and the limit of detection were not investigated. Second, this study lacks clinical performance, which is crucial for evaluating the developed Cas12a system compared to real-time PCR, widely regarded as the gold standard for acute CHIK viral infection.

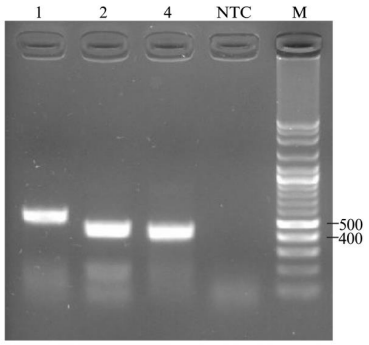

Figure 3. Agarose gel electrophoresis of RPA products by amplification of extracted CHIKV genomic RNA with the following pairs of primers: 1) CH2320F-CH2R, 563bp; 2) CH2555F-CH2R, 428bp; 3) CH2320F-CH2693R, 405bp. M: DNA Marker.

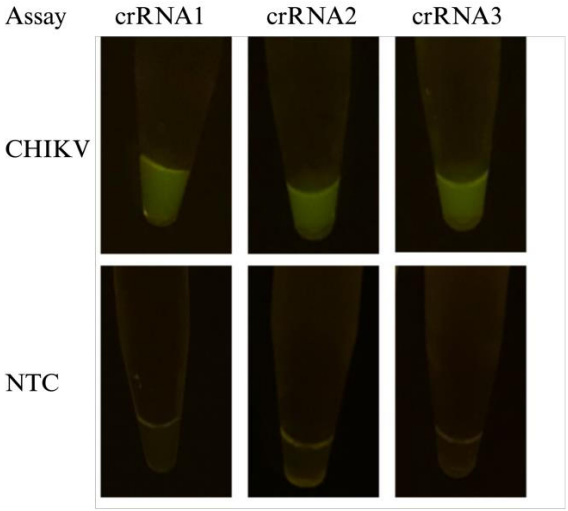

Figure 4. Detection of CHIKV-based CA12a assay. RPA coupled CRISPR-Cas12a assay with crRNA1, crRNA2, and crRNA3 on CHIKV. NTC: no template.

Conclusion

The results of this study demonstrate that the designed RPA primers and Cas12a crRNAs successfully detect the CHIKV gene, providing significant preliminary evidence for a promising diagnostic approach that retains the ability to detect all CHIKV genotypes.

Further study will determine the limitations and suggestions addressed, as well as the assay's sensitivity, specificity, positive predictive value, negative predictive value, and accuracy. This newly developed Cas12a-based assay will be a promising point-of-care diagnostic tool for acute chikungunya infection.

Acknowledgements

This study was supported by the scholarship program for ASEAN or Non-ASEAN countries, supported by Chulalongkorn University. Funding was provided in part by Ratchadapiseksompotch Endowment Fund (GA68/016), Faculty of Medicine, Chulalongkorn University.

Conflict of interest statement

The authors declare there is no conflict of interest in this study.

Data sharing statement

The datasets generated or analyzed during the current study are included in this published article. Additional supporting data are available from the corresponding author upon reasonable request.

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