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Construction and Characterization of an Infectious Clone of Chikungunya Virus Strain 37997

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Abstract: Chikungunya virus (CHIKV) is the pathogen that causes Chikungunya fever, and there is no specific antiviral drug treatment for CHIKV infection. A simple and efficient CHIKV infectious clone is an important tool for studying the mechanism of CHIKV infection and development of vaccine and drug. Based on the cDNA sequence of the virus published in GenBank, we obtained the whole sequence of the viral genome by cleavage and ligation of adjacent fragments, and inserted a mammalian intron sequence which does not affect the production of the virus to ensure the stable inheritance of the virus. We introduced an EcoR I restriction endonuclease cleavage site and a T7 promoter sequence at the 5' end, and introduced a Not I restriction endonuclease cleavage site at the 3' end. Then the entire sequence was inserted into pVRC-L vector to obtain the infectious clone pCHIKV. The transfected supernatant was obtained by transcribing and transfecting into BHK-21 cells. The virus was identified by sequencing of the transfected supernatant, empty plaque, indirect immunofluorescence and mice infection test. The results showed that pCHIKV and the transfected supernatant were sequenced as expected, the viral titer was calculated as $5*10^7$ FFU/ml, the virus could infect Vero E6 cells, the virus-infected mice became ill on the 3rd day, and all of them died on the 4th day, and the virus were present in the liver tissues of the dead mice. These results indicated the successful construction of the CHIKV infected clone.

1. Introduction

CHIKV, first discovered in Tanzania in 1952, which is an important group of alphaviruses, it mainly transmitted by Aedes aegypti mosquitoes, and is generally susceptible to the population [1-2]. The locals call it chikungunya, which means "to become curved" [3], to describe the patient's swollen joints and curved body posture. In addition to causing symptoms such as fever, muscle pain, and skin rashes, it can often cause chronic arthritis that lasts for more than one year, and it has been reported that CHIKV can cause more serious illnesses in recent years, such as encephalitis and hemorrhagic disease [4]. Currently, CHIKV has spread to more than 100 countries and regions

around the world [5], with a cumulative total of 10 million suspected and confirmed CHIKV cases worldwide, and imported cases have been reported in China regularly [6-7]. There is no specific treatment for CHIKV infections, which poses a significant burden to global public health.

The CHIKV genome is an unsegmented, positive-sense, single-stranded RNA with a total length of 12 kb approximately, including a 5' end cap structure (m7GpppAm), two non-coding regions (NCR), two open reading frames (ORFs), a poly (A) tail at the 3' end, and there is a subgenomic promoter (SP) that regulates the expression of structural gene between non-structural and structural gene [8]. The non-coding region of viruses plays a regulatory role in the process of viral replication and translation. The 5' end ORF encodes four non-structural proteins: nsP1, nsP2, nsP3, nsP4, which are mainly responsible for the replication and transcription of the viral genomic RNA. The 3' end ORF encodes viral structural proteins: capsid protein (Capsid), envelope proteins E1, E2 and E3. The envelope surface glycoproteins E1 protein are responsible for participating in the membrane fusion process of the virus [9].

Viral infectious clone, a plasmid containing the full-length viral genome namely, is an important tool for using in basic virology research and antiviral science and technology development. Viral cDNA instability caused by mutations, deletions, or rearrangements in the viral genome, resulting in noninfectious clones, is the most common problem encountered in the development of reverse genetic systems for many RNA viruses. In this study, we constructed an infectious clone plasmid pCHIKV that can stably inherit the full length of CHIKV using Escherichia coli amplification and can be used to rescue the 37997 strain of CHIKV which has high titers, can infect Vero E6 cells, and can infect lethal type I interferon receptor knockout mice.

2. Materials and methods

2.1. Strains, plasmids and mice

Receptor STBL3 bacteria were purchased from Shanghai Weidi Biological Company, plasmid vector pVRC-L was processed and kept by the Biomedical Protection Department of Naval Medical University (EcoR I cleavage site and Not I cleavage site were added to the 5' and 3' ends of the NIH plasmid vector pVRC respectively), and type I interferon receptor knockout mice (A129 mice) were maintained and bred by the Biomedical Protection Faculty of the Naval Medical University [10].

2.2. Reagents

Fetal bovine serum (FBS), Dulbecco' modified Eagle' medium (DMEM), ethylene diamine tetraacetic acid (EDTA), penicillin, streptomycin and glutamine were purchased from Gibco; *EcoR* I, *Spe* I, *Nhe* I, *Nsi* I restriction endonuclease, T4 DNA ligase, and HisScribe^{TM}T7 mRNA transcription kit were purchased from New England Biolabs; The mRNA purification reagent, transfection reagent Lipofectamine^{TM}2000 reagent, RNA extraction reagent Trizol Regent and anti-mouse IgG containing fluorescein Alexa Fluor 488-labeled were purchased from Thermo Fisher, the monoclonal antibody containing anti-CHIKV envelope E1 protein was purchased from R&D Systems.

2.3. Cell culture

BHK-21 cells and Vero E6 cells were kept by the Biomedical Protection Department of Naval Medical University, these cells were cultured in DMEM complete medium containing 10% FBS, 1% glutamine, 1% penicillin and 1% streptomycin at 37℃ in a 5% CO_2 incubator. The fluid was changed every 2 days. When the cell fusion reached 90%, the passaged cells were digested with

trypsin containing EDTA.

2.4. Chemical synthesis of CHIKV gene fragments

Based on the sequence of a strain of CHIKV published by GenBank (GenBank: EU224270.1), the A nucleobase located at 8213 nt was mutated to G nucleobase, then the nucleobase sequence at genome 8210-8213 nt was changed from CAGA to CAGG, which formed the optimized splice site CAG/G of the intron in mammalian cells without changing the coding amino acid, referring to the mammalian cells Expression vector pCI-neo (Promega product, GenBank: U47120.2) in the intron sequence, inserted a segment of intron gene sequence. The genome was divided into five fragments (Figure 1A) using four enzymatic sites in the CHIKV whole genome sequence, and the T7 phage RNA polymerase binding site (TAATACGACTCACTATAG) was introduced at the 5' end of fragment 1, and the enzyme cleavage sites at the junctions of each fragment were retained. The *EcoR* I cleavage site and *Not* I cleavage site were introduced at the 5' and 3' ends of each fragment, and each segment was inserted into the *EcoR* I cleavage site and *Not* I cleavage site of pVRC-L vector. The five plasmids were synthesized by Shanghai generay biotechnology company.

2.5. Splicing of gene fragments

Slicing fragment 1 plasmid with *EcoR* I and *Spe* I enzyme, isolating fragment 1 by agarose gel electrophoresis, recovering fragment 1; Slicing fragment 2 plasmid with *EcoR* I and *Spe* I enzyme, isolating linearized fragment 2 plasmid by agarose gel electrophoresis, recovering linearized fragment 2 plasmid; ligating the recovered fragment 1 and linearized fragment 2 plasmid with T4 DNA ligase, and ligand reaction product Transform the susceptible STBL3, culturing STBL3 with 3-5 ml of LB culture medium, extracting the plasmid, digest it with *EcoR* I and *Spe* I enzyme, and analyzing whether fragment 1 is inserted into the fragment 2 plasmid; if fragment 1 was digested, then fragment 1 and fragment 2 were successfully ligated. Five gene fragments were ligated in a similar way (Figure 1A). The plasmid inserted with the full-length CHIKV genome containing the intron sequence was named pCHIKV. The theoretical CHIKV gene expression structure in the pCHIKV plasmid is shown in Figure 1B (China National Microbiological Sciences Data Center, No: NMDCN0002PGG). The plasmid pCHIKV was transformed into STBL3, the plasmid was extracted, and then STBL3 was transformed again for 10 times, and the plasmid extracted from each extraction was sent to Shanghai Biosune Biological Company for gene sequencing to detect whether there were any nucleotide mutations, and to verify the genetic stability of the plasmid.

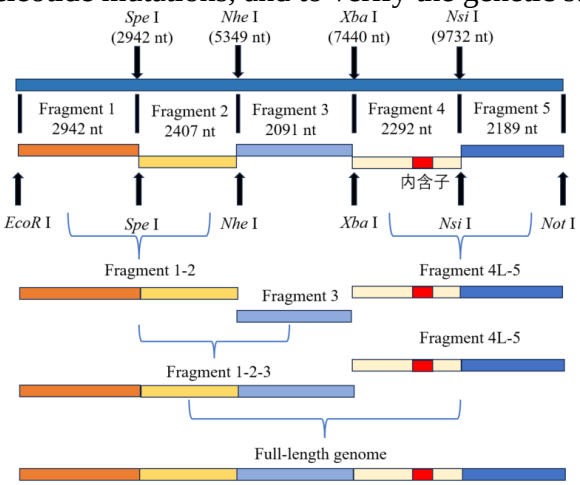

Figure 1A: Splicing method of the full-length genome of CHIKV.

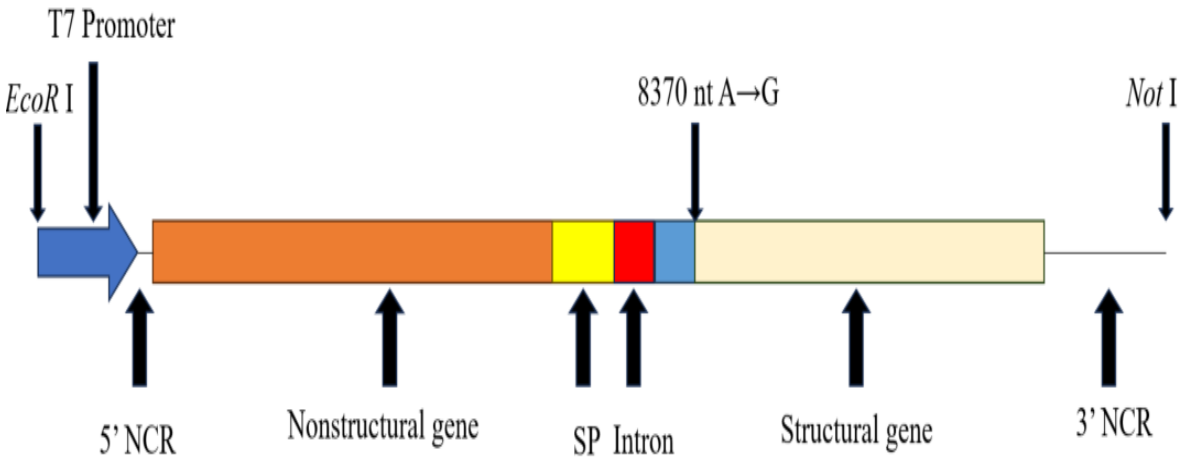

Figure 1B: Expression structure of CHIKV gene in CHIKV infectious clone plasmid.

2.6. Obtaining cell cultures containing CHIKV virus

The *Not* I digested linearized plasmid pCHIKV was used as a template, and CHIKV genomic RNA was transcribed using the T7 RNA in vitro transcription kit. The mRNA with 7-methyl guanosine (m7G) cap structure at the 5' end was obtained by one-step co-transcription and capping. The transcription reaction solution was purified using magnetic bead mRNA purification reagent and the concentration of the mRNA was determined by NanoDrop microspectrophotometer. BHK-21 cells were inoculated into 24-well plates, and on the following day, when the cell density was about 80%, the cells were transfected with the prepared CHIKV genomic RNA, and 2 μg of CHIKV RNA and 2 μl of Lipofectamine™ 2000 reagent were added to each well. After 8 h of transfection, the cell culture medium was aspirated, and 500 μl of medium was added to each well to continue incubation for 48 h. The cell culture medium was aspirated and frozen in -80 ℃ refrigerator.

2.7. Empty spot assay

Vero E6 cells were inoculated into 24-well plates, and at 95% confluence, 500 μl of CHIKV RNA transfected BHK-21 cell culture medium collected by gradient dilution was added to each well, and incubated at 37 ℃ for 2 h. Then the viral supernatant was removed, and 1 ml of Sodium Carboxymethylcellulose mixture (containing 4% Sodium Carboxymethylcellulose, 2 x DMEM (containing 4% sodium carboxymethylcellulose, 2 x DMEM culture medium and fetal bovine serum in the ratio of 50:48:2) was added to each well to continue incubation. The size of empty spots was observed under the microscope, and the mixture was completely aspirated when there was obvious empty spot formation. Each well was stained with 200 μl of crystal violet for 15 min, rinsed with water, and the plaques were counted, and the viral titer was calculated by the Reed-Muench method.

2.8. Indirect immunofluorescence assay

The collected cell culture solution was inoculated with Vero E6 cells at a density of about 80-90% in a 96-well plate at a 10^4-fold dilution infection. 20 h later, the culture solution was aspirated, 100 μl of methanol was added to each well, and fixed in a refrigerator at -20 ℃ for 20 min. PBS was used to wash the methanol, and then 100 μl of 3% BSA- PBS was added to each well and

placed in a horizontal shaker for 1 h. The 3% BSA-PBS was aspirated, washed three times with PBS, and 100 μl monoclonal antibody containing anti-CHIKV envelope E1 protein (1000-fold dilution of the antibody) was added to each well and placed on a shaker for 1 h. The working solution of the antibody was aspirated, washed three times with PBS, and 100 μl 1% BSA-PBS containing fluorescein Alexa Fluor 488-labeled anti-mouse IgG (1500-fold dilution of fluorescein antibody) was placed on a shaker at room temperature and avoided light and shaken slowly for 1 h. Fluorescein antibody working solution was aspirated, PBS was washed 3 times, and DAPI cell nucleus staining solution was added to each well at 100 μl, and shaken slowly for 10 min at room temperature and avoided light in the The cell nuclei were observed and photographed under a fully automated cell imaging and analysis system (BioTek Cytation 5 Imaging Reader, BioTek, USA).

2.9. Mice infection test

Ten 6-week-old A129 mice were divided into two groups of 5 mice each, and each one of the experimental group was intraperitoneally injected with 200 μl dose of $1*10^4$ FFU of BHK-21 cell culture solution; the control group was intraperitoneally injected with an equal volume of BHK-21 cell culture solution not transfected with CHIKV RNA. The mice were kept for 14 days and observed. The liver tissues of diseased and dead mice were removed, ground and homogenized, and frozen at -80 ℃ for spare.

2.10. Statistical analysis

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3. Conclusions

3.1. Sequencing identification of infectious clone and virus

The plasmids from 10 extractions were sequenced and compared with the sequences from the China National Microbiology Data Center (No. NMDCN0002PGG), the sesults showed genome sequence is consistent, indicating the initial success in constructing an infectious clone of CHIKV. Second-generation sequencing of RNA from culture fluid of extracted transfected BHK-21 cells and mouse liver tissues homogenate was performed, and the results were compared with the sequence of the GenBank virus strain (GenBank: EU224270.1), it was consistent with the original sequence of CHIKV except for the G nucleobase at 8213 nt, it did not contain mammalian intron sequences, and did not have any base mutations, insertions or deletions.

3.2. Identification of virus cytotoxicity

The viral titer was calculated to be $5*10^7$ FFU/ML by the viral etch assay (Figure 2A). Immunofluorescence was used to detect whether the viral proteins were expressed in Vero E6 cells, and a distribution of green fluorescence was seen in infected cells, whereas no green fluorescence was seen in uninfected cells (Figure 2B), indicating the presence of CHIKV envelope protein E1 in Vero E6 cells.

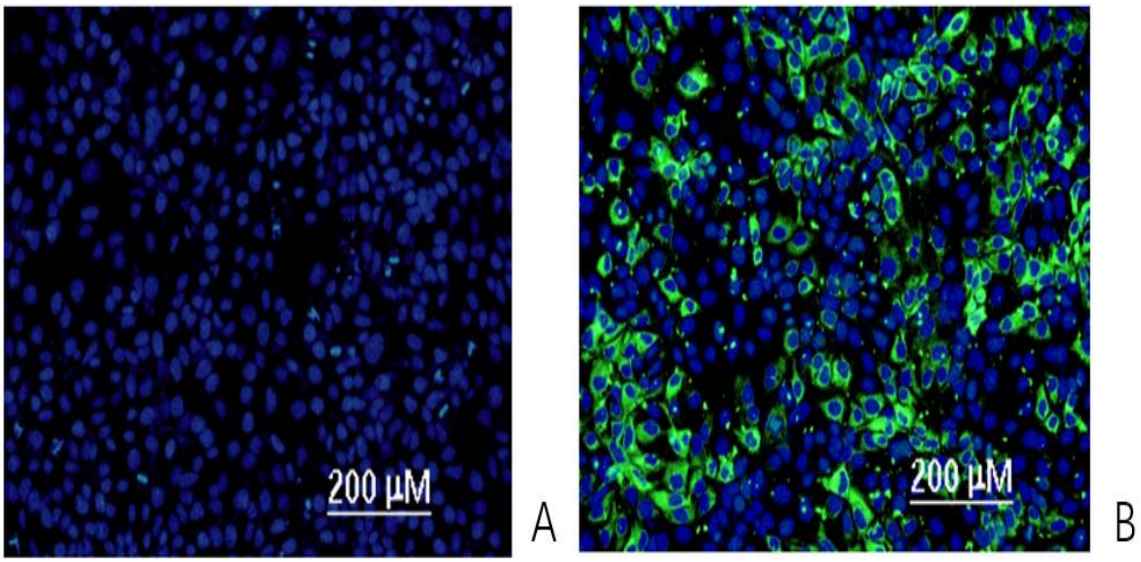


Figure 2A: Determination of CHIKV titers in BHK-21 cell cultures by the empty spot method.

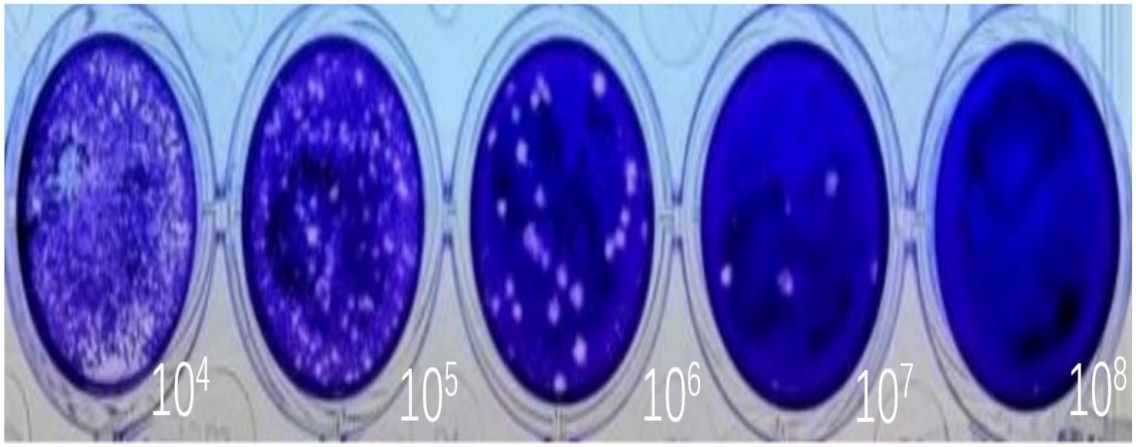
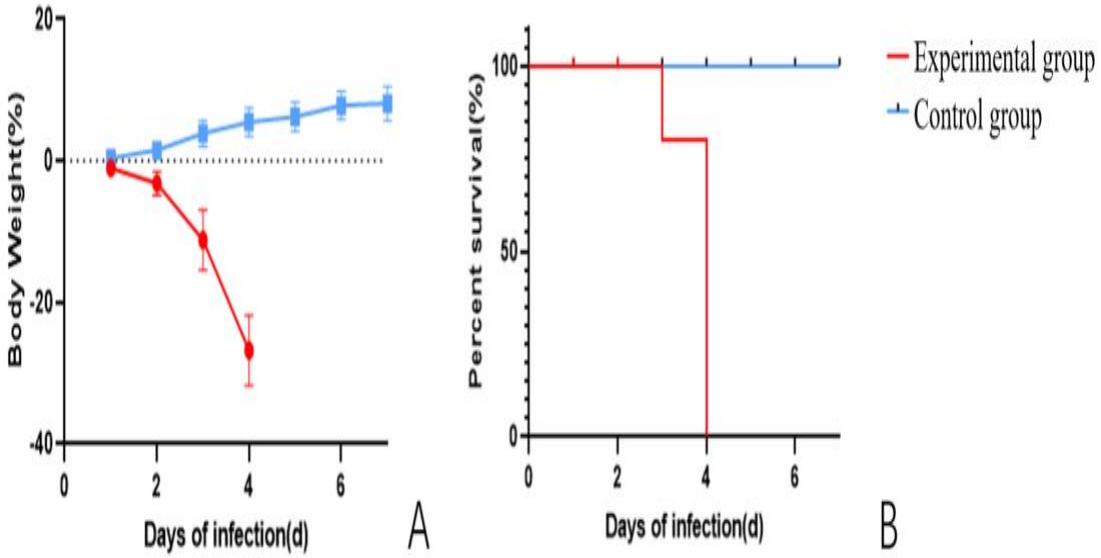


Figure 2B: Immunofluorescence detection of CHIKV envelope E1 protein in Vero E6 cells.A: control BHK-21 culture medium-infected Vero E6 cells; B: RNA-transfected BHK-21 culture medium-infected Vero E6 cells.

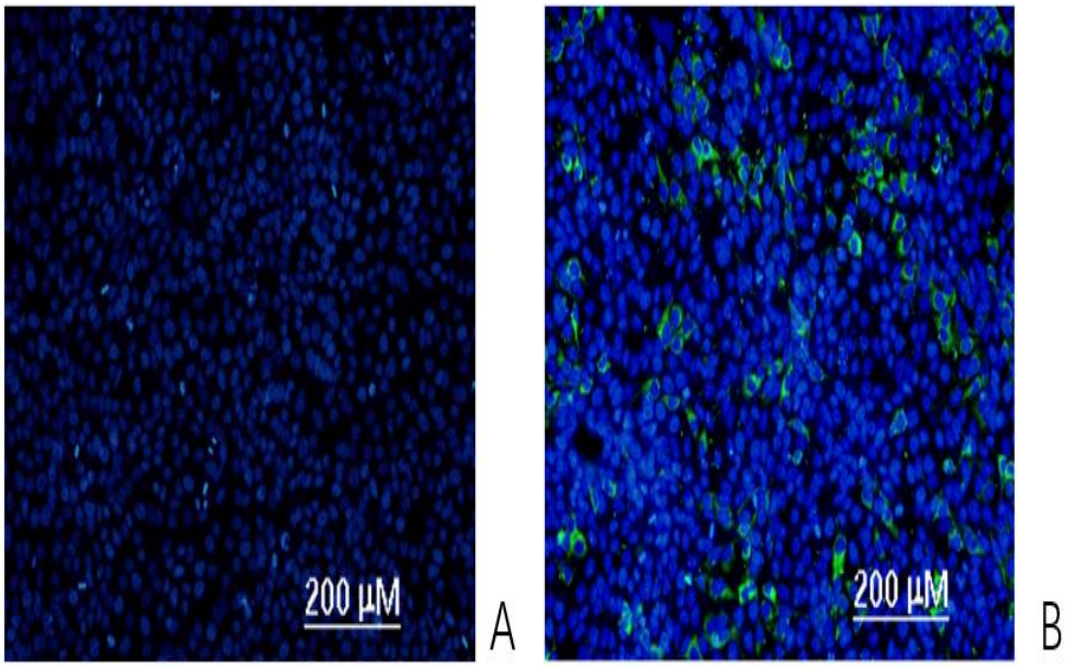
3.3. Characterization of virus pathogenicity in mice

A129 mice were inoculated with the virus by intraperitoneal injection, and PBS was injected intraperitoneally as a control to test the pathogenicity of the virus in mice. The mice in the experimental group showed symptoms such as slow movement, arched backs, and vertical hairs on the 3rd day, and all of them died on the 4th day (Figure 3A). The liver tissues of the dead mice and a portion of the control mice were ground and homogenized, and the supernatant of the homogenate was infected with Vero E6 cells, which were detected by immunofluorescence after 20 h. The experimental group showed green fluorescence under the fluorescence microscope, while the control group showed no green fluorescence (Figure 3B). All mice in the control group were healthy and alive for 14 days, except for two mice that was used to prepare liver tissue homogenate and was executed.



(A) Body weight change curve of virus-infected mice.
(B) Survival curve of virus-infected mice.

Figure 3A: Pathogenicity of CHIKV in A129 mice.



(A) Control mouse liver tissue homogenate-infected Vero E6 cells.
(B) CHIKV-infected mouse liver tissue homogenate-infected Vero E6 cells.

Figure 3B: Immunofluorescence detection of CHIKV envelope E1 protein in liver tissue homogenate-infected Vero E6 cells of virus-infected mice.

4. Discussion

Many RNA viruses encounter problems in the development of reverse genetic systems due to the fact that the virally encoded products are toxic to the bacterial host during propagation of the plasmid vector in the bacterial host cells. One of the common ways to overcome this problem is to use medium or low copy number plasmid vectors and suitable bacterial host strains. In this study, fragment 4 and fragment 5 ligation reaction solutions without insertion of mammalian intron sequences were transformed into Escherichia.coli several times without positive colonies, and it was considered that there may be an Escherichia.coli promoter at the interface of fragment 4 and fragment 5, which leads to the viral genome expressing abnormal proteins in Escherichia.coli that are toxic to Escherichia.coli. We chose to mutate a base in the CHIKV genome to form an optimized shear site for mammalian cell introns, and inserted a segment of mammalian intron gene sequence to serve as a segment of interfering gene to abort the viral genes to transcriptionally express toxic proteins in Escherichia.coli, while excision of the intron sequence did not affect the production of CHIKV in mammalian cell mRNA. The results showed the successful construction of an efficient and convenient CHIKV infectious clone, which provides an important idea for constructing viral infectious clones.

CHIKV can be categorized into four strains based on genetic differences: the West African strain (WA), the East/Central/South African strain (ECSA), the Asian strain (Asian) and the Indian Ocean lineage (IOL) [11]. A single amino acid mutation in the viral structural protein E1 enhances viral fitness in the more widely distributed Aedes albopictus mosquito, making CHIKV more transmissible, and is largely responsible for the large-scale spread of CHIKV in India, Europe and the Americas [12]. In this study, we constructed a full-length infectious clone of West African CHIKV strain 37997, which can be used as an important tool to study the effect of CHIKV amino acid mutations on viral pathogenicity and adaptation.

Acknowledgements

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