Chapter 16
Pseudotyped Viruses for the *Alphavirus* Chikungunya Virus

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Abstract Members of the genus *Alphavirus* are mostly mosquito-borne pathogens that cause disease in their vertebrate hosts. Chikungunya virus (CHIKV), which is one member of the genus *Alphavirus* [1], has been a major health problem in endemic areas since its re-emergence in 2006. CHIKV is transmitted to mammalian hosts by the *Aedes* mosquito, causing persistent debilitating symptoms in many cases. At present, there is no specific treatment or vaccine. Experiments involving live CHIKV need to be performed in BSL-3 facilities, which limits vaccine and drug research. The emergence of pseudotyped virus technology offered the potential for the development of a safe and effective evaluation method. In this chapter, we review the construction and application of pseudotyped CHIKVs, the findings from which have enhanced our understanding of CHIKV. This will, in turn, enable the exploration of promising therapeutic strategies in animal models, with the ultimate aim of developing effective treatments and vaccines against CHIKV and other related viruses.

Keywords Chikungunya virus · Pseudotyped virus · Neutralization

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Abbreviations

BSL	Biosafety level
CHIKF	Chikungunya fever
CHIKV	Chikungunya virus
ECSA	East-Central-Southern African
HIV	Human immunodeficiency virus
MLV	Murine leukemia virus
MXRA8	Matrix remodeling-related protein 8
ORF	Open reading frame
PBNA	Pseudotyped virus-based neutralizing antibody assay
PRNT	Plaque reduction neutralization test
UTR	Untranslated region
VSV	Vesicular stomatitis virus

16.1 Biological Characteristics of Chikungunya Virus

Viruses belonging to the *Alphavirus* genus can infect humans and often cause symptoms such as joint pain and fever. According to the latest revision report published by the International Committee on Taxonomy of Viruses (ICTV) in 2021, the *Alphavirus* genus comprises 32 species, including Aura virus, Barmah Forest virus, Bebaru virus, Caaingua virus, Cabassou virus, Chikungunya virus (CHIKV), Sindbis virus, Eastern equine encephalitis virus, Western equine encephalitis virus, Mayaro virus, Semliki Forest virus, and Onyong-nyong virus.

CHIKV is an arthropod-borne *Alphavirus* that is transmitted to humans primarily via the bite of an infected mosquito. CHIKV is becoming widespread in Africa, South Asia, and Southeast Asia, with high infection rates [2, 3]. Infection of humans by CHIKV can cause Chikungunya fever (CHIKF), an acute febrile illness associated with severe, often debilitating, polyarthralgias, fever, rash, and even death [4–6]. CHIKV has emerged in more than 100 countries, with approximately one million people infected each year. In 2017, the World Health Organization (WHO) reported 10 potential infectious diseases for priority research, with CHIKV on the list.

16.1.1 Molecular Structure

CHIKV is an arbovirus of the *Alphavirus* genus, which belongs to the *Togaviridae* family [7]. It is a single-stranded positive-sense RNA virus with a full-length genome of 11.8 kb, including 5′ and 3′ untranslated regions (UTRs) and two open reading frames (ORFs) (Fig. 16.1) [8]. One ORF encodes four non-structural
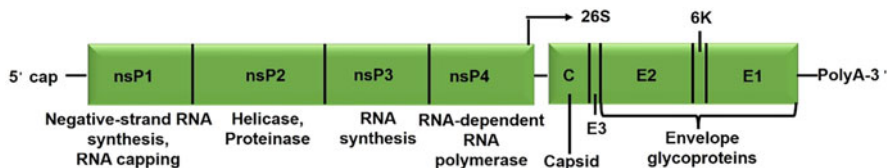
Fig. 16.1 Schematic diagram of the CHIKV genome structure

proteins, namely, nsP1, nsP2, nsP3, and nsP4; the other ORF encodes five structural proteins, namely, capsid proteins C, E3, E2, 6 K, and E1 [9].

Capsid protein C includes two functional domains: an N-terminal RNA binding domain and a C-terminal protease domain. The former binds to genomic RNA, while the latter is involved in the assembly of structural proteins [10, 11]. E1 contains a hydrophobic fusion loop, and under acidic conditions, the CHIKV membrane protein undergoes a conformational change, exposing the fusion loop and promoting nucleocapsid release [12]. Glycoprotein E2 binds to receptors on the host cell membrane to form an endosome and enter the cytoplasm [13, 14]. E3 prevents premature fusion of the heterodimer formed by E2 and E1 with the cell membrane [15]. Studies have shown that two amino acid residues, Gly91 and His230, play a key role in the membrane fusion of E1 [16]. 6 K is the signal peptide of glycoprotein E1 and consists of only 55–60 amino acids [15, 17]. Recently, through structural analysis, Chinese scholars found that the matrix remodeling-related protein 8 (MXRA8) molecule widely distributed on the surface of chondrocytes, muscle cells, and skeletal muscle cells is the receptor of CHIKV, which confirmed the mechanism of host cell invasion by CHIKV and provided a new target for the research and development of vaccines and broad-spectrum neutralizing antibodies [18–20].

16.1.2 Genotypes and Variants

According to genetic analysis, CHIKVs comprise one serotype and three genotypes, namely, the West African genotype, the East-Central-Southern African (ECSA) genotype, and the Asian genotype [21].

Sequence analysis of the isolated viruses revealed that the re-emerging strains of CHIKV originating from Reunion Island belonged to the ESCA genotype [22, 23], while the strains currently circulating in Southeast Asia include both Asian and ESCA genotypes [24–27]. The strains introduced into St. Maarten and the Americas belong to the Asian genotype [24, 25, 28]. Some evidence suggests that the CHIKV strain isolated in Brazil in 2014 has ESCA genes similar to those circulating in Angola [29].

Numerous studies have shown that the large-scale CHIKF outbreak in Reunion Island in 2005–2006 was caused by a CHIKV strain (ECSA genotype) transmitted

via *Aedes albopictus*. This CHIKV epidemic strain harbors a A226V mutation on the E1 protein, which changes the transmission host of CHIKV from *Aedes aegypti* to *A. albopictus* [30–33].

In 2009, a new ECSA subtype (GenBank accession no. HM159390) was discovered in Hyderabad, India, with a K211E mutation in the E1 protein (E1-K211E) [34]. In 2010, a CHIKV strain containing two novel mutations E1-K211E and E2-V264A was found among all CHIKV isolates from New Delhi, India [35]. In 2011 and 2012, the abovementioned epidemic strains (E1-K211E and E2-V264A) were found in Tamil Nadu and Kolkata, respectively [34, 36].

16.1.3   Pathogenic Mechanisms and Biosafety Risk

CHIKV can be cultured on a variety of passage cell lines, such as C6/36, AP61, Vero, LLC- MK2, and BHK-21 cells [37]. CHIKV can also be isolated and cultured in vivo by intracranial inoculation of suckling rats aged 1–3 days.

CHIKV enters the host's skin 24–48 hours after a mosquito bite, resulting in viremia. Viremia manifests as a systemic proinflammatory response associated with leukopenia and mononucleosis. The virus circulates through the blood and attacks fibroblasts in muscles and joints, macrophages in lymphoid tissue, and endothelial cells in the liver [12, 38, 39].

CHIKV is considered to cause a high level of individual harm but a low level of group harm. Nucleic acid and serological testing of *A. albopictus*-inactivated serum and frozen specimens can be performed in biosafety level (BSL)-2 laboratories, but potential and confirmed CHIKV-positive samples have to be handled in BSL-3 facilities. It is important that researchers adhere to strict safety precautions to avoid unnecessary biosafety risks.

16.2   Construction of Pseudotyped CHIKV

Currently, there are three commonly used packaging backbone vectors for pseudotyped CHIKVs; these are human immunodeficiency virus (HIV-1)-based lentiviral vectors, vesicular stomatitis virus (VSV)-based vectors, and murine leukemia virus (MLV)-based vectors. In addition, when the abovementioned packaging backbone vector cannot construct a pseudotyped virus, a recombinant pseudotyped virus can be constructed by reverse genetics technology.

16.2.1 Construction of Pseudotyped CHIKV Using Different Vectors

16.2.1.1 Lentiviral Vectors

The HIV-1-based lentiviral system is the most commonly used packaging system for the construction of pseudotyped CHIKVs. As early as 2017, our group constructed the West African genotype pseudotyped CHIKV using the HIV-1 system [40]. The full-length CHIKV West African genotype (strain 37,997, GenBank accession no. AY726732) structural protein (C-E3-E2-6K-E1) was synthesized, and after codon optimization in mammalian cells, it was cloned into the eukaryotic expression vector pcDNA3.1(+), which constituted the CHIKV membrane protein expression plasmid pCMV3.1-CHIKV. Then, 293 T cells were transfected with the appropriate ratio (1:2) of membrane protein expression plasmid pCMV3.1-CHIKV and backbone plasmid pSG3Δenv.cmv.Fluc, and the supernatant was collected after 48 hours. The final prepared CHIKV pseudotyped virus titer was 1.0×10^7 $TCID_{50}$/ml. In 2019, Korean scholars constructed a pseudotyped CHIKV containing two reporter genes based on a lentivirus system [41]. In this study, a three-plasmid system was used to co-transfect 293 T cells with a full-length CHIKV ECSA genotype (strain KNIH/2009/77, GenBank accession no. MN158171.1), structural protein (C-E3-E2-6K-E1), expression plasmid pCMV2-FLAG-CHIKVst, a lentivirus-based dual vector (Luc2P-pLVX-IRES-ZsGreen1), and a packaging vector of lentivirus reporter (psPAX2). The supernatant was harvested to obtain the pseudotyped virus, CHIKVpseudo. There are two important findings from this study. First, when constructing the membrane protein expression plasmid, FLAG tag was added to aid the subsequent detection of protein expression. Second, the system contains two reporter genes, encoding luciferase and GFP. The tracer results of the two reporter genes can be compared simultaneously.

The packaging process of pseudotyped CHIKV based on lentiviral vectors is generally conducted as follows:

1. Seed 293 T cells one day before transfection.
2. Incubate the cells with DMEM (high glucose) containing 10% FBS for 12–18 h. The cells should be 80% confluent before transfection.
3. Co-transfect CHIKV structural protein expression plasmid and lentivirus-based packaging vector plasmid/plasmids using Lipofectamine 3000 or other transfection reagents. Dilute the plasmid with Opti-MEM if necessary.
4. Incubate at 37 °C for 4–6 h.
5. Remove the medium, add fresh DMEM with 10% FBS, and incubate at 37 °C overnight for another 42–44 h.
6. Harvest the culture supernatant. Filter using a 0.45 μM pore-size filter and store at ·70 °C until use.

All work involving pseudotyped CHIKV was performed in a BSL-2 facility.

16.2.1.2 VSV-Based Vectors

A pseudotyped CHIKV based on the VSV vector system has also been constructed [42]. Researchers constructed a West African genotype (strain 37,997, GenBank accession no. AY726732, E3-E2-6K-E1) CHIKV pseudotyped virus based on the VSV vector containing the GFP reporter gene and the firefly luciferase reporter gene, respectively, for the subsequent study of the virus entry mechanism.

The packaging process of pseudotyped CHIKV based on VSV vectors is generally conducted as follows:

1. Seed 293 T cells into a 10-cm dish one day before transfection.
2. Incubate the cells with 10 ml of DMEM (high glucose) containing 10% FBS. The cells should be 80% confluent before transfection.
3. Remove the medium, wash the cells once with the medium, and then add 5 ml of fresh medium.
4. Transfect 5 μg of CHIKV structural protein expression plasmid using Lipofectamine 3000 or other transfection reagents. Dilute the plasmid with Opti-MEM if necessary.
5. Incubate at 37 °C for 6 h.
6. Remove the medium, add fresh DMEM with 10% FBS, and incubate at 37 °C overnight for another 18 h.
7. Inoculate with a multiplicity of infection of 1.0 of VSVΔG/GFP*-G, VSVΔG/ Luci*-G, or VSVΔG/SEAP*-G.
8. Adsorb the viruses at 37 °C for 6–8 h.
9. Remove the medium and wash with phosphate-buffered saline (PBS) or serum-free DMEM three times.
10. Add 10 ml of the medium and incubate at 37 °C overnight (17–18 h).
11. Harvest the culture supernatant. Filter using a 0.45 μM pore-size filter and store at ·70 °C until use.

All work involving pseudotyped CHIKV was performed in a BSL-2 facility.

16.2.1.3 MLV-Based Vectors

Like HIV-1, MLV is also a retrovirus that is commonly used as a pseudotyped viral packaging vector. French researchers constructed pseudotyped CHIKV using an MLV vector. Briefly, 293 T cells were co-transfected with the pTG5349 MLV packaging plasmid, the pTG13077 plasmid encoding an MLV-based vector containing a CMV-GFP internal transcriptional unit, a plasmid encoding the Gag-pol proteins of MLV, and a plasmid encoding the CHIKV La Réunion infectious clone (strain LR2006 OPY1, GenBank accession no. KT449801.1) viral envelope glycoproteins (C-E3-E2-6K-E1) [43]. After 24 hours of co-transfection with plasmids, the packaging effect of pseudotyped virus can be determined by observing the fluorescence signal intensity of GFP.

According to published studies and our previous research, all three genotypes of CHIKV can successfully be used to construct pseudotyped viruses. For pseudotyped CHIKV, the expression of E protein can be improved by removing capsid protein C, but whether or not the full-length structural protein is selected, the E2 and E1 protein genomes are essential for the construction of pseudotyped CHIKV [44].

16.2.2 CHIKV Infectious Clones and Virus-like Particles

Compared with other viruses, alphaviruses are more suitable for the construction of infectious clones and virus-like particles. In addition to pseudotyped viruses, several CHIKV infectious clones expressing different reporter genes have been developed to study viral replication and pathogenic mechanisms. The insertion position of the reporter gene in the *Alphavirus* genome is critical for the stability of infectious clones and the virulence of animals [45].

A common strategy for constructing *Alphavirus* infectious clones is to initiate reporter gene expression via an additional subgenomic promoter upstream of the true subgenomic promoter (5'-26S) or the E1 protein (3'-DP) downstream. Compared with 3'-DP, the 5'-26S method is more stable with minimal loss of viral virulence [45, 46]. A reproducible CHIKV infectious clone containing a near-infrared fluorescent protein (iRFP) reporter gene—CHIKV-iRFP—was developed, which was used to establish in vivo imaging of small animals. Briefly, the *iRFP* gene was fused to the full-length CHIKV genome (Asian lineage, GenBank accession no. KC488650) between nsP4 and the capsid (C), and another subgenomic (SG) T7 promoter was introduced downstream of the *iRFP* gene to facilitate the replication of recombinant viruses [47].

16.3 Application of Pseudotyped CHIKV

16.3.1 Neutralizing Assay Based on Pseudotyped CHIKV

The main application of pseudotyped CHIKV is to establish a pseudotyped virus-based neutralizing antibody assay (PBNA). The process of pseudotyped CHIKV infection of cells can be blocked by neutralizing antibodies in test samples. After a test sample interacts with the pseudotyped CHIKV, detecting the expression of the reporter gene carried by the pseudotyped virus can reflect the titer of the neutralizing antibody to be tested. Compared with plaque reduction neutralization test (PRNT), this method has the advantages of high safety, simple operation, easy access, and potential high throughput. In addition, PBNA allows for a reduction in the biosafety level of CHIKV neutralizing antibody detection (from BSL-3 to BSL-2), which was comparable to the detection results obtained with the live virus method.

16.3.1.1 Correlation of PBNA and PRNT

Chung et al. constructed a pseudotyped CHIKV based on a lentiviral vector and established a stable PBNA using the pseudotyped virus [41]. The activity of CHIKV E2 antibody was detected using this method, as well as using classical PRNT. The results showed that the use of PBNA yielded a mean Log IC_{50} value of 5.85 ± 0.09 pg/ml, while the use of PRNT yielded a Log IC_{50} value of 5.68 ± 0.06 pg/ml. The neutralization assay of CHIKVpseudo showed a CV value of 1.09%, similar to the PRNT CV value of 0.98%. In conclusion, PBNA using CHIKVpseudo is a safe, rapid, and reliable neutralization assay that can be used to evaluate the neutralizing activity of serum or monoclonal antibody against CHIKV.

16.3.1.2 Factors Closely Related to CHIKV PBNA

Undoubtedly, a high titer of pseudotyped virus reliably provides PBNA results. However, other factors can also affect the normal operation of PBNA.

(a) **Pseudotyped virus-infected cells:** In general, irrespective of the vector used to construct pseudotyped CHIKV, cells susceptible to pseudotyped virus should be selected as target cells for PBNA. Pseudotyped CHIKV has a wide range of cell tropism [48], and 293 T cells [40, 48] and HeLa cells [41] can be used as infective cells in PBNA. In addition, the source of cells should be clear and the number of passages should be controlled. For experiments with longer cell culture times (more than 48 hours), the evaporative effect in edge wells should be considered. When establishing this method, the cell seeding concentration range should be experimentally validated and optimized.

(b) **Negative and positive controls:** A perfect PBNA should have negative and positive controls to ensure the accuracy of the test results. If international or domestic standards cannot be obtained, the positive serum obtained after animal immunization can be used as a positive control.

(c) **Amount of CHIKV pseudotyped virus:** Adding too much virus can result in false negatives or can cause the inhibition rate to reverse the concentration gradient, which will affect the experimental results.

(d) **Incubation time for CHIKV pseudotyped virus:** The co-culture time of cells and pseudotyped CHIKV should be selected according to the specific characteristics of the pseudotyped virus. For example, the co-culture time for PBNA-based VSV vector is generally 24 h, while for the PBNA-based HIV backbone, the co-culture time is generally 48–72 h.

(e) **Cut-off value:** To determine the critical values for the negative and positive controls (cut-off value), the serum of the negative group with a similar level of background should be selected as the source of the negative sample for establishing the cut-off value. Generally, the test sample size should not be less than 100. The experiment should be repeated at least three times, and

appropriate methods should be used to determine the cut-off value, such as using the geometric mean $\pm$ 2SD or 3SD as the final cut-off value.

16.3.2 Establishment of an in Vivo Imaging Model of Small Animals

The pseudotyped virus can be used to establish a visual dynamic monitoring model in mice, to study the tissue distribution after virus infection, and to evaluate the in vivo efficacy of monoclonal antibodies, small molecule inhibitors, and vaccines. Using the accurate quantitative performance and imaging characteristics of the system, the infection site of pseudotyped virus in animals can be dynamically observed, the degree of infection can be judged according to the luminescence intensity and location, and the effect of monoclonal antibodies, drugs, and vaccines can be evaluated [49–51].

Our group infected mice with a HIV-1 vector-based pseudotyped CHIKV, and after optimizing variables, such as the mouse strain, infection route, and detection time, finally established a visual CHIKV pseudotyped virus infection model. Subsequently, the passive immunization effect of antiserum and the active immunization effect of the DNA vaccine were evaluated in vivo using the established mouse infection model [40].

16.3.3 Use of a Capture Antigen in CHIKV IgM Detection

The reference method for the detection of CHIKV-specific IgM is the IgM capture enzyme-linked immunosorbent assay (MAC-ELISA). In the MAC-ELISA method, CHIKV viral lysate is usually used as the antigen bound to the specific IgM to be detected. However, obtaining viral lysates is time-consuming and requires BSL-3 facilities. Chemical treatments such as beta-propiolactone must be used to inactivate the virus before use. However, beta-propiolactone treatment may alter the overall structure of enveloped viral proteins, which may modulate the affinity of antibodies against these proteins, thereby reducing the sensitivity of detection [52, 53].

To overcome these challenges, researchers used pseudotyped CHIKV as a substitute for CHIKV viral lysate [54]. The purified and lyophilized pseudotyped CHIKV was directly applied to paper-based analytical devices as the coating antigen, and the CHIKV IgM antibody detection results could be obtained within 8–10 min. The results showed that the sensitivity of this method to detection using patient serum was 70.6% and the specificity was ~98%. While these results are promising, they are only preliminary, and more extensive validation with a greater number of samples is needed before pseudotyped viruses can replace traditional antigens in serological tests.

16.3.4   Drug Screening

Until now, treatment for CHIKF has been mainly symptomatic because of the lack of an FDA-approved vaccine or effective antiviral drugs. One of the reasons for the delay in the development of anti-CHIKV drugs is the need for BSL-3 facilities in which to conduct the research, which limits the potential for high-throughput operations. Using pseudoviruses to screen small-molecule compounds that have already been approved allows for rapid drug repositioning. In addition, pseudotyped viruses can also be used to screen traditional Chinese medicines with antiviral activity, all of which have the advantages of high throughput and low cost [55, 56].

German scholars determined the anti-CHIKV activity of curcumin or *Boswellia serrata* gum resin extract (AKBA) using pseudotyped CHIKV based on a lentiviral vector [57]. Both compounds blocked the entry of pseudotyped CHIKV and inhibited pseudotyped CHIKV infection in vitro. The IC_{50} values were 6.75 μM for AKBA and 10.79 μM for curcumin.

16.3.5   The Mechanism of Viral Infection

An increasing number of reports have shown that pseudotyped viruses play an important role in the study of virus invasion mechanisms. Pseudotyped viruses have the advantages of being safe, easier to manipulate, easier to construct or discover mutations in coat genes, and easier to quantify the process of entry. In addition, the viral envelope protein itself can be studied using pseudotyped viruses, and the entry process of viral replication can be studied separately from other steps. Pseudotyped viruses are mainly used to study virus infection tropism, cell tropism, receptor recognition, and the virus entry mechanism in vitro [58, 59].

There is evidence that CHIKV enters host cells via a clathrin-dependent endocytic pathway [60]. Endosomal acidification inhibitors attenuate CHIKV infection. Therefore, it is believed that CHIKV particles bind to cell surface receptors and are internalized into the host cell endosome, and then endosomal acidification promotes fusion of the virus with the endosomal membrane through the CHIKV envelope (E) protein. Researchers used MLV vector-based pseudotyped CHIKV to infect 293 T and TE671 cells and found that virus particles containing the CHIKV E protein entered 293 T cells by endocytosis but were internalized into TE671 cell vesicles by macrophage phagocytosis [44]. Through vesicle acidification, the conformation of the E protein is altered, thereby activating its ability to promote membrane fusion. In addition, it was found that digestion of E protein by endosomal cathepsin B more efficiently promoted membrane fusion activity and enhanced infection.

16.4   Conclusion

The ongoing advances in the pseudotyped virus construction system have further confirmed it as an effective virological research strategy. Although some limitations of this system still need to be taken into account, for example, pseudotyped viruses cannot fully simulate the pathogenesis of live virus infection [61], and the innovative virus packaging system is needed for application.

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