UNIVERSIDAD AUTÓNOMA DE SAN LUIS POTOSÍ

FACULTAD DE MEDICINA

Centro de Investigación en Ciencias de la Salud y Biomedicina (CICSaB)

"Construcción de plásmidos del virus de Chikungunya, de replicación y ayuda para la transfección de células de mamíferos."

TESIS QUE PRESENTA

M. C. MAYRA COLUNGA SAUCEDO

PARA OBTENER EL GRADO DE DOCTORA
EN CIENCIAS BIOMÉDICAS BÁSICAS

DIRECTOR DE TESIS
DR. MAURICIO COMAS GARCIA

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CREDITOS INSTITUCIONALES

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Tesis que presenta:

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viruses

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Construction of a Chikungunya Virus, Replicon, and Helper Plasmids for Transfection of Mammalian Cells

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Abstract: The genome of Alphaviruses can be modified to produce self-replicating RNAs and virus-like particles, which are useful virological tools. In this work, we generated three plasmids for the transfection of mammalian cells: an infectious clone of Chikungunya virus (CHIKV), one that codes for the structural proteins (helper plasmid), and another one that codes nonstructural proteins (replicon plasmid). All of these plasmids contain a reporter gene (mKate2). The reporter gene in the replicon RNA and the infectious clone are synthesized from subgenomic RNA. Co-transfection with the helper and replicon plasmids has biotechnological/biomedical applications because they allow for the delivery of self-replicating RNA for the transient expression of one or more genes to the target cells.

Keywords: Chikungunya virus; alphavirus DNA vectors; replicon plasmid; helper plasmid

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1. Introduction

Chikungunya virus (CHIKV) belongs to the *Togaviridae* family and genus *Alphavirus*, and it is also known as an Arbovirus (Arthropod-borne virus) because it is transmitted by mosquitos from the genus *Aedes*. According to the European Centre for Disease Prevention and Control, in 2022, there were 338,592 confirmed cases and 70 deaths associated with CHIKV. The majority of cases were reported in Brazil (240,344), India (93,113), Guatemala (1435), Thailand (775), and Malaysia (662). A meta-analysis of 44 studies worldwide that included 51,599 people from 29 countries revealed that up to 25% of the population could be seropositive to CHIKV. The region with the highest prevalence was Southeast Asia, with 42%, and the lowest was the Eastern Mediterranean, with 2% [1]. CHIKV has a positive-sense, single-stranded RNA genome (gRNA) that is encapsulated in an T = 4 icosahedral virion. The virion is composed of a nucleocapsid containing 240 monomers of capsid protein, one copy of the gRNA, a lipid bilayer, and eighty trimers of a heterodimer of the glycoproteins E2/E1. The capsid proteins and glycoproteins interact with each other and assemble into a virion with icosahedral symmetry (both at the core and virion level) with a diameter between 50 and 70 nm [2]. The gRNA (11.5 kb) is divided into the 5′ and 3′UTR, an intergenic region, and two open reading frames (ORF1 and 2). The ORF1 encodes four nonstructural proteins (nsP1234), which are part of the RNA-dependent RNA polymerase complex (RdRp) and contains the sequence for a putative packaging signal. The ORF2 encodes the structural proteins C, E3, E2, E1, TF, and 6K [2,3]. An advantage of the gene

organization of Alphaviruses is that it allows for the replacement of the ORF2 by one or more non-viral sequences, giving rise to a self-replicating RNA called replicon that amplifies the gene of interest but does not produce viral particles. In addition, deletion of the ORF1 results in a defective helper RNA that, under certain circumstances, can generate structural proteins, but it is unable to self-replicate [4–6]. When the helper RNA is in a mammalian expression plasmid and depending on how the ORF1 was deleted, the amplification of the helper RNA can depend either on the RdRp (Replicon-dependent helper) or on the cellular polymerases (Replicon-independent helper) [7–11]. In other words, deletion in the ORF2 results in a self-replicating RNA that does not give rise to virions (i.e., replicon RNA), while deletion of the ORF1 results in an RNA that will produce virus-like-particles, but it cannot be self-amplified (i.e., helper RNA).

The need to generate new therapies against autoimmune, infectious, and non-communicable diseases has resulted in the development of new strategies for gene expression. Most systems that allow for the delivery and expression of heterologous genes in cell cultures, animal models, and humans are based on viral vectors derived from DNA viruses and retroviruses. The most common are Adenovirus, Lentivirus, Herpesvirus, and Adeno-associated viruses [12–17]. However, while these vectors are extremely promising, they have some disadvantages caused by their limited tropism and the high prevalence of similar viruses, resulting in pre-existing immunity that decreases the efficacy of the viral vector. In addition, in some cases, there may be a risk (although of low probability) of integration of the viral genome into the host genome [18]. These are some reasons that have motivated the development of alternative gene delivery systems; the best examples are micelles and lipid vesicles containing mRNAs [19]. A disadvantage of these lipid/mRNA-based systems is the infrastructure required for transport and long-term storage before reconstitution (e.g., ultra-low temperature freezers at −80 °C) [20].

It is in this context that the generation of a replicon/helper system derived from an Alphavirus is an appealing approach. For example, the genome can be easily modified to generate single-round viral vectors [21–24]. This system can be used to produce viral particles that are structurally identical to the parental virus that contain a gene of interest instead of structural proteins [11,25]. The structural proteins account for about 4.6 kilobases; hence, they could be replaced by several genes, which can be separated into several individual proteins by using several subgenomic promoters and/or by fusing them to peptides, such as P2A [5,26,27]. Further, CHIKV has a very wide tropism because it can use multiple receptors and cellular factors, such as Actin gamma 1, collagen type I-alpha-2, and PTPN2, PHB1, Mxra8, DC-SIGN, TIM-1, and glycosaminoglycans [28–33]. Furthermore, given the fact that the Old and New-World alphaviruses have different tropisms, it is possible to exchange the glycoproteins of an alphavirus that produced dengue fever, such as disease (e.g., CHIKV) for one that results in encephalitis (e.g., Venezuelan equine encephalitis virus) and, thus, tropism of the viral vector [34]. It is also important to point out that the seroprevalence in the human population against this virus in places within non-tropical climates is low compared to other viruses [35]. In cases where the seroprevalence against a particular Alphavirus was to be high, the glycoproteins of this viral vector could be exchanged for ones of another alphavirus with the same tropism but with low seroprevalence in such a region. Nonetheless, the seroprevalence of CHIKV is mostly limited to the tropical areas of the Americas, the Indian subcontinent, and southeast Asia; the rest of the world has a seroprevalence between 0 and 2% [1].

One disadvantage of most Alphavirus helper/replicon systems is that, in most cases, single-round infectious particles are generated by the co-transfection or co-electroporation of in vitro transcribed RNAs, which is not optimal for the large-scale production of viral vectors. Therefore, there is a need to generate CMV-driven single-round infectious CHIKV particles that could be affordable compared to those that are based on transfection with RNA.

Over the course of more than two decades, alphaviruses have been studied to understand how their genome is organized, how they replicate and assemble, and their infection

mechanism; however, there is still no approved vaccine or specific treatment against this pathogen [36–41]. There are several limitations that have lagged behind the development of alphavirus-based therapies or vaccines. For example, using electroporation to introduce in vitro transcribed viral RNAs into insect or mammalian cells is expensive and impractical for massive administration. Further, the RNA can be easily degraded and scaling up its production can be challenging. Nonetheless, Alphavirus replicon/helper systems represent a promising approach for immunization and protection against pathogenic viruses and gene therapy [17,42–45]. However, in order for this technology to become a reality, we need to move the helper/replicon systems from using in vitro transcribed RNAs to plasmids for mammalian cell expression (e.g., pVax1 or pcDNA3.1, which in both cases generate mRNAs using the cellular nuclear machinery thanks to the presence of a cytomegalovirus (CMV) RNA polymerase II promoter and simian vacuolating virus 40 (SV40) and β-globin polyA signals). In fact, it has been shown that plasmid-based replicon vectors produce higher levels of encoded heterologous proteins compared to conventional DNA vaccines and provide an affordable platform [46–49]. It is important to point out that there are several plasmids that contain the full-length CHIKV genome either under an SP6 or T7 in vitro transcription promoter [50–54] or under a CMV promoter [54–57]. Moreover, most replicon/helper systems require transfection or electroporation of in vitro transcribed RNA rather than transfection with a CMV-driven plasmid [7].

Here, we report the generation of a plasmid for expression in mammalian cells that contains the full-length genome of the attenuated strain 181/25 of the Chikungunya virus under the CMV promoter and two transcription terminators and polyA signals. In this plasmid, the viral 3′ UTR and polyA were maintained intact by inserting the hepatitis delta virus ribozyme. Further, this clone expresses the mKate2 protein as a reporter gene. Transfection of HEK-293T cells with this plasmid results in particles able to infect Vero E6 and HEK-293T cells. Ultrastructural analysis of the infected cells reveals virions and type-I and -II cytopathic vesicles identical to those observed with the wild-type virus. Furthermore, we generated two more expression plasmids for mammalian cells that encode for either the ORF1 (replicon) or the ORF2 (Replicon-independent helper); thus, we were able to generate a single-round infectious virus. This study demonstrates that Chikungunya DNA vectors can be easily modified to have the potential to study assembly (i.e., by using the replicon-independent helper plasmid), how this virus replicates its genome (i.e., by using the replicon plasmid), to generate single-round infectious particles to understand the infectious cycle, to determine the neutralizing activity of antibodies, or for gene delivery.

2. Materials and Methods

2.1. Plasmid Constructions

The original plasmid used to generate helper and replicon plasmids contained the full-length genome of the attenuated strain 181/25 of the Chikungunya virus in a vector for in vitro transcription with the SP6 RNA polymerase (SP6-CHIKV). First, the original plasmid was modified to change the SP6 transcription promoter to a BamHI restriction site by site-directed mutagenesis using primers SP6toBamHI-F and SP6toBamHI-R (see Supplementary Table S1) and a polymerase Phusion HS II (ThermoFisher Scientific, Waltham, MA, USA). The PCR product was digested with DpnI (ThermoFisher Scientific) and directedly used to transform NEB 5-α-competent cells (New England Biolabs, Ipswich, MA, USA). The resulting colonies were grown in LB media with ampicillin (Caisson Laboratories, North Logan, UT, USA), and the plasmids were purified using the alkaline lysis method [58]. The modified plasmid, as well as the pVax1 plasmid (ThermoFisher Scientific, Waltham, MA, USA), were digested with NotI and BamHI (New England Biolabs, Ipswich, MA, USA) enzymes. The digested pVax1 was treated with alkaline phosphatase (New England Biolabs, Ipswich, MA, USA), then both plasmids were ligated with a T4 DNA ligase (New England Biolabs, Ipswich, MA, USA) and were transformed into NEB 5α cells. The resulting colonies were grown in LB media with kanamycin (Caisson Laboratories, North Logan, UT, USA).

The replicon-independent helper plasmid (pVax-Help) was generated by deleting the non-structural proteins 1–4 (161–7510 pb) from pVax-CHIKV. First, the plasmid was linearized by inverse PCR using the primers DelNsP4-F and DelNsP1-R (Table S1). Then, the plasmid was re-circularized using the KLD kit (New England Biolabs, Ipswich, MA, USA) and directly transformed into NEB 5α cells. The resulting colonies were grown in LB media with kanamycin.

The replicon plasmid pVax-Rep was generated by two inverse PCR/re-circularization consecutive protocols as described above using the pVax-CHIKV plasmid as a template. First, the capsid gene was deleted (7571–8355 pb) by inverse PCR using the primers DelCPE3-F and DelCPE3-R (Table S1), re-circularized with the KLD kit, and transformed into NEB 5α cells. The resulting plasmid was used as a template to delete the E3 to E1 genes (9058–12,072 pb) using the primers DelFMtoE1-F and DelFMtoE1-R (Table S1), ligated with the KLD kit, and transformed into NEB 5α cells.

The full-length CHIKV genome and the replicon sequences were moved from the pVax vector to a pACNR1811 plasmid [59]. The viral sequence was inserted between the CMV promoter and the hepatitis delta virus (HDV) sequence. The original viral polyA was conserved during this step. This mutagenesis was performed using the In-Fusion Cloning Kit method (Clontech, Mountain View, CA, USA). The pACNR1811 lineal vector was inverse-PCR amplified using the primers ICD-F and ICD-R. Then, the viral sequences were amplified by PCR with the primers Chik-F and Chik-R (Table S1). All PCR products were DpnI-digested, column-purified, UV-Vis-quantified, and ligated following the vendor's protocol. The resulting plasmids were called pACNR-CHIKV and pACNR-Rep.

All plasmids were SANGER-sequenced (LANBAMA-IPICyT, Mexico). The annotated sequences are in the Supplementary Information and the electronic files are freely available upon request.

2.2. Cell Culture Conditions

HEK-293T (CRL-3216) cells were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). HEK-293T cells were grown and maintained in Dulbecco's modified Eagle medium (DMEM; Corning, Manassas VA, USA). The culture medium was supplemented with 10% fetal bovine serum (FBS; Gibco, Thermo Fisher Scientific, Waltham, MA, USA). Vero E6 cells (CRL-1586) (ATCC, Manassas, VA, USA) were grown in DMEM (Corning, Manassas VA, USA) and supplemented with 2% FBS (Gibco, Thermo Fisher Scientific, Waltham, MA, USA). All cell lines were grown in a 5% CO_2 atmosphere at 37 °C using culture tissue-treated plates and flasks (Corning, Manassas VA, USA).

2.3. Plasmid Transfection

Infectious CHIKV particles were produced by transfecting HEK-293T cells with the pACNR-CHIKV plasmid. The single-round infectious particles and virus-like-particles (VLPs) were produced in HEK-293T cells (producer cells) by either co-transfecting them with the pACNR-Rep and pVax-Help plasmids or just pVax-Help, respectively. The cells were seeded in a 6-well dish at a cell density of 250,000 cells/well. Twenty-four hours later, the cells were transfected with lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA) reagent. The viral particles and VLPs were collected at 24 and 48 h post-transfection (h.p.t.), centrifuged at 1000 RFC for 10 min, and frozen at −80 °C. The expression of mKate2 and cell morphology were analyzed by fluorescence microscopy Lionheart FX Automated Microscope (BioTek Instruments, Winooski, VT, USA) using a Texas RED and DAPI filters, as well as a high-contrast phase mode. All the DNAs used for transfection were purified with the kit MaxiPrep (Promega Corporation, Fitchburg, WI, USA).

Expression Kinetics of mKate2 Protein Reporter with Chikungunya Plasmid

HEK-293T cells were transfected with 0.5 μg and 1 μg of pACNR-CHIKV following the above-described protocol. The fluorescence signal was measured in a fluorescence

microscopy Lionheart FX Automated Microscope using a Texas RED filter (termed as mKate2 in the figures) for 96 h every 12 h.

2.4. Entry Assay

The entry of the viral particles and VLPs was performed by overlaying 165 µL of the supernatant of the producer cells on HEK-293T cells seeded in a 12-well dish at a cell density of 100,000 cells/well, which were previously washed to remove the FBS. Two hours post-infection (h.p.i.), 1 mL of fresh media was added to the cells, and 24 h.p.i., the expression of mKate2 and Hoechst stain was analyzed in a fluorescence microscopy Lionheart FX Automated Microscope.

2.5. CHIKV Infection and Viral Quantification by Plaque Assay

Vero E6 cells were infected with the supernatants obtained from cultures subjected to the transfection previously described with pACNR-CHIKV. The viral progeny virus was collected at 24 and 48 h post-infection and filtered through a 0.22 µM syringe filter and frozen at −80 °C. The quantification of the virus was performed by overlaying 450 µL of the supernatant of the infected cells on Vero E6 cells seeded in a 6-well dish at a cell density of 400,000 cells/well, which were previously washed to remove the serum. Two hours h.p.i., 3 mL of 1% Carboxymethylcellulose (Sigma-Aldrich, Saint Louis, MO, USA) was added to the cells and incubated. After 4 days, the cells were stained with crystal violet solution for visualization and counting of plaques [60]. The supernatant of mock-infected cells was used as a negative control.

2.6. Ultra-Structural Analysis by Thin-Section Transmission Electron Microscopy

Monolayers of cells were either infected with CHIKV at a MOI of 1 or co-transfected with pACNR-Rep and pVax-Help. Then, 24 h-post-infection and 48 h.p.t., the cells were fixed with a solution of 4% formaldehyde, 0.05% picric acid, and 0.1 M sodium cacodylate, pH 7.4. The cells were processed following the protocol from Tobin et al. 1996 [61]. The samples were washed with EMBed 812 resin and polymerized for 48 h at 55 °C. The resin was cut with an EMUC7 ultramicrotome into 70 nm-thick slices, which were mounted on copper TEM grids and stained with uranyl acetate and lead citrate. Samples were analyzed on a JEM-2100 transmission electron microscope (JEOL Ltd., Akishima, Tokyo, Japan) at 200 kV using a Gatan OneView 4K camera.

3. Results

3.1. Generation of Viral, Replicon, and Helper CHIKV Plasmids for Expression in Mammalian Cells

The plasmid SP6-CHIKV is used to produce CHIKV via electroporation of in vitro transcribed RNA with SP6 polymerase; hence, we decided to transfer the CHIKV genome from an SP6-based vector to a CMV-driven plasmid for mammalian expression (i.e., pVax1). Since the pVax1 plasmid has its own polyadenylation signal, the viral polyA tail was removed. Then, we mutated the pVax-CHIKV plasmid to produce two more plasmids: the helper plasmid was generated by deleting non-structural proteins (nsP1234) and the replicon plasmid was obtained by deleting capsid protein and E1-E2-6K-E1 proteins (Figure 1A).

Transfection of HEK-293T cells with pVax-CHIK (data not shown) and pVax-Rep (Figure S1) did not result in the detection of the reporter gene mKate2. However, transfection with pVax-Help results in the expression of mKate2, as seen by the red signal in the mKate2 channel (Figure S1). The transcription of pVax-Help mRNA depends on cellular polymerase, while mRNA transcription of pVax-CHIKV and pVax-Rep depends on cellular polymerase and viral RNA-dependent RNA polymerase (RdRp). In order to test if the polyadenylation signal from the pVax affected the functionality of the viral sequences, the CHIKV genome and the replicon RNA were moved from pVax to the plasmid pACNR, which contains an HDV ribozyme after the viral polyA tail (Figure 1A). Figure 1B shows that the transfection of HEK-293T cells with pACNR-CHIKV resulted in the expression of

the reporter gene mKate2 (red cells) and that most cells are mKate2-positive. This signal indirectly shows expression of the viral proteins from the subgenomic RNA (i.e., SGP in Figure 1A). The kinetics of the transfection was monitored by measuring the number of mKate2-positive cells using an inverted fluorescence microscope. Figure 1C shows that the transfection with 1 µg resulted in a higher expression of mKate2 at early times (before 48 h.p.t.) compared to the cells transfected with 0.5 µg. However, at around 48 h.p.t., the amount of mKate2-positive cells was higher in the cultures transfected with 0.5 µg of DNA than 1.0 µg. After 72 h.p.t., the total number of cells and mKate2-positive cells decreases and the integrity of the cell monolayer disappears.

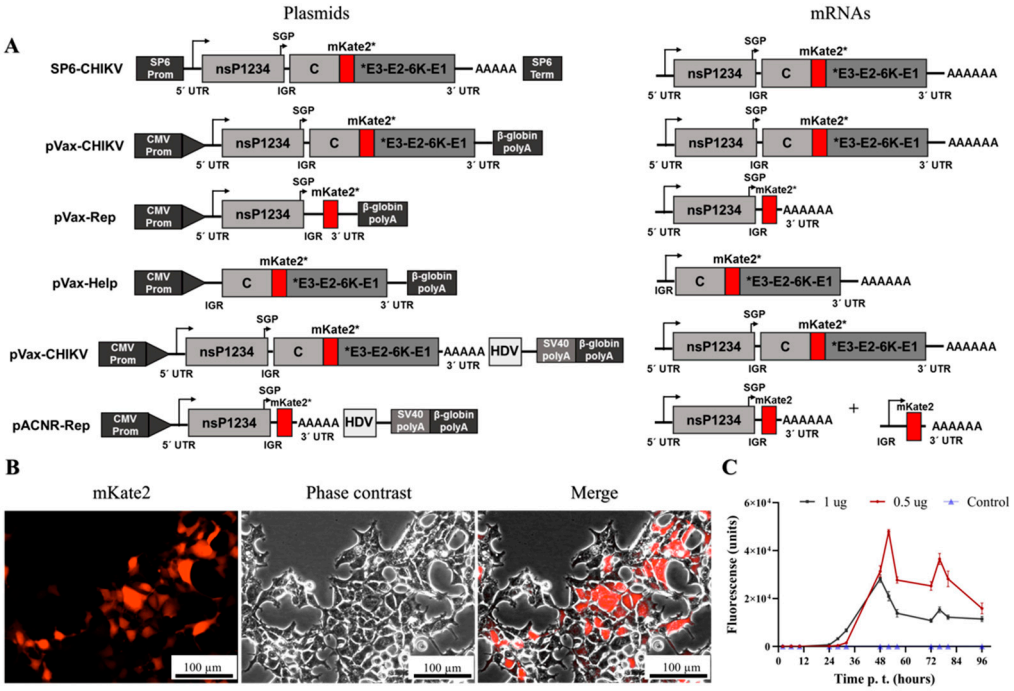

Figure 1. Construction and evaluation of an expression plasmid for CHIKV. (**A**) Schematic representation of the plasmids generated here. The SP6-CHIKV plasmid is used for in vitro transcription with SP6 polymerase and contains the full-length genome of the attenuated strain 181/25 of the Chikungunya virus. The genomic CHIKV was inserted into the pVax-CHIKV vector from which the human cytomegalovirus (CMV) immediate early enhancer and promoter sequences and βGH polyA derived from pVAX. The SV40 polyA and hepatitis delta virus (HDV) were inserted, and the genome was moved from the pVAX to the vector pACNR1811. The asterisk means that mKate2 is a reporter gene. (**B**) Micrographs of HEK-293T cells transfected with the pACNR-CHIKV plasmid in a fluorescent field (mKate2 channel), phase contrast, and a merge of both. (**C**) Kinetics of the expression of mKate2 from the plasmid pACNR-CHIKV. Bar, 100 µm.

3.2. Viral Production and Analysis of Infection by Microscopy

To corroborate that the particles obtained by transfecting cells with pACNR-CHIKV are infectious, the supernatant of the transfected cells was used to infect HEK-293T and Vero E6 cells. Figure 2A shows the fluorescence micrographs of HEK-293T infected to MOI of 1; the red signal indicates the cells that are transfected and the blue signal indicates the cell nuclei. This data demonstrate the expression of the reporter gene located in the subgenomic RNA. Figure 2A also shows the same for Vero E6 cells; however, by comparing the number of mKate2-postive cells compared to the number of nuclei, it seems that the infection efficiency in Vero E6 cells is lower than in HEK-293T (Figure 2A). Nonetheless, Figure 2B demonstrates that the infected Vero E6 cells exhibited the cytopathic effects characteristic of CHIKV infection; the cells change from barely visible by light microscopy to round cells that refract light. Figure 2C shows the typical plaques from this infection

in Vero E6 cells. From the plaque assays in Vero E6, we determined that the cumulative production of CHIKV was 1.77 × 10^7 PFU/mL and 2.21 × 10^7 PFU/mL at 24 h.p.i. and 48 h.p.i., respectively (Figure 2D). The data suggest that transfection of mammalian cells, such as the HEK-293T with the pACNR-CHIKV plasmid, results in infectious virions that can infect HEK-293T and Vero E6 cells.

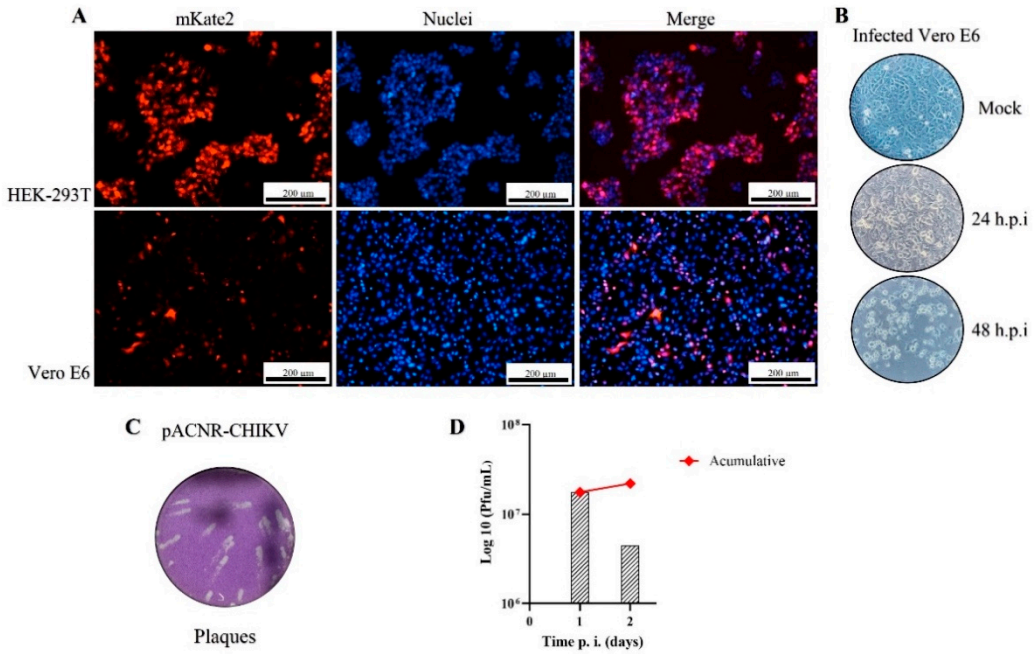

Figure 2. The Chikungunya virus plasmid pACNR-CHIKV produces a viral infection. (**A**) HEK-293T and Vero E6 cells infected with the supernatant of the producer (transfected) cells in fluorescent-field mKate2 expression (Texas Red filter), nuclei stain (DAPI filter), and merge at MOI of 1 for 24 h.p.i. (**B**) Cytopathic effects induced by the CHIKV infection in Vero E6 cells at MOI of 1. (**C**) CHIKV plaques on Vero E6 cells stained with crystal violet and (**D**) and virus titer measured as plaque-forming units (PFUs). Bar, 200 µm.

Figure 3A shows viral particles, released from the cell as well as budding particles, with their characteristic double membrane and protrusion from the E2/E1 proteins (see triangle). This figure also shows budder cores at the plasma membrane where they interact with the glycoproteins so that the virion can be fully assembled and can then bud out. Figure 3B shows an accumulation of cores in the cytoplasm near the endoplasmic reticulum. Figure 3C shows the core particles before interacting with the glycoproteins that are not budding yet. Finally, Figure 3D shows the classic type-II cytopathic vacuoles. In this vacuole, the viral particles surround the central vacuole, while traveling to the plasma membrane. All the virions present the characteristic morphology of CHIKV with an approximate diameter of 50 nm (Figure S2).

3.3. The Replicon and Helper Vector System Generates Single-Round Infectious Particles with Gene Delivery Capability

The next step was to test whether the plasmid pACNR-Rep results in the expression of the fluorescent gene mKate2 (Figure 4A). This plasmid cannot give rise to infectious particles because it does not contain structural genes. Figure 4B shows fluorescence micrographs (mKate2), phase contrast, and a merge of them of HEK-293T cells transfected with pACNR-Rep, pVax-Help, and pACNR-Rep/pVax-Help. The expression of the reporter gene, generated from the subgenomic RNA, shows that both plasmids are functional. Subsequently, HEK-293T cells were co-transfected with both plasmids; this resulted in the fluorescence intensity and the number of co-transfected fluorescent cells being higher than when the cells were transfected with only one of the two plasmids.

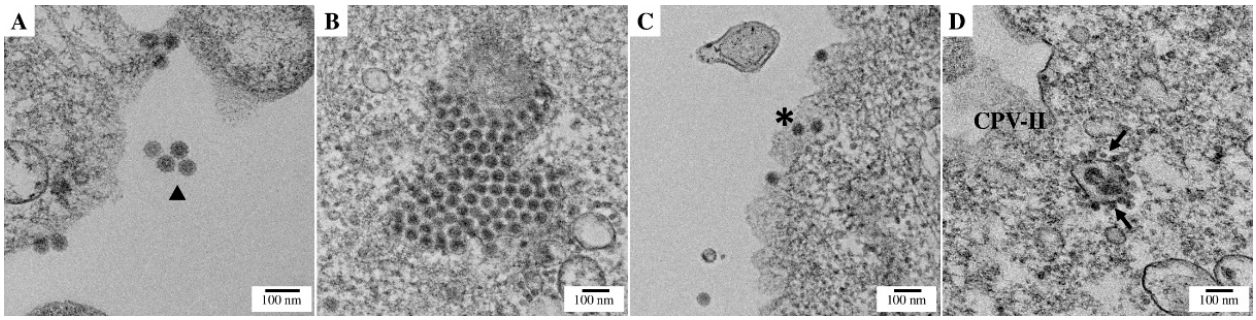

Figure 3. Chikungunya virus pACNR-CHIKV plasmid induces replication and assembly of viral infectious particles. Vero E6 cells infected with Chikungunya virus at a MOI of 1 were assessed at 24 h post-infection. TEM images show Chikungunya (**A**) four extracellular virions (▲), (**B**) a viral factory, (**C**) four virions budding from a membrane (*), and (**D**) type-II cytopathic vacuoles (arrow, CPV-II). Bar, 100 nm.

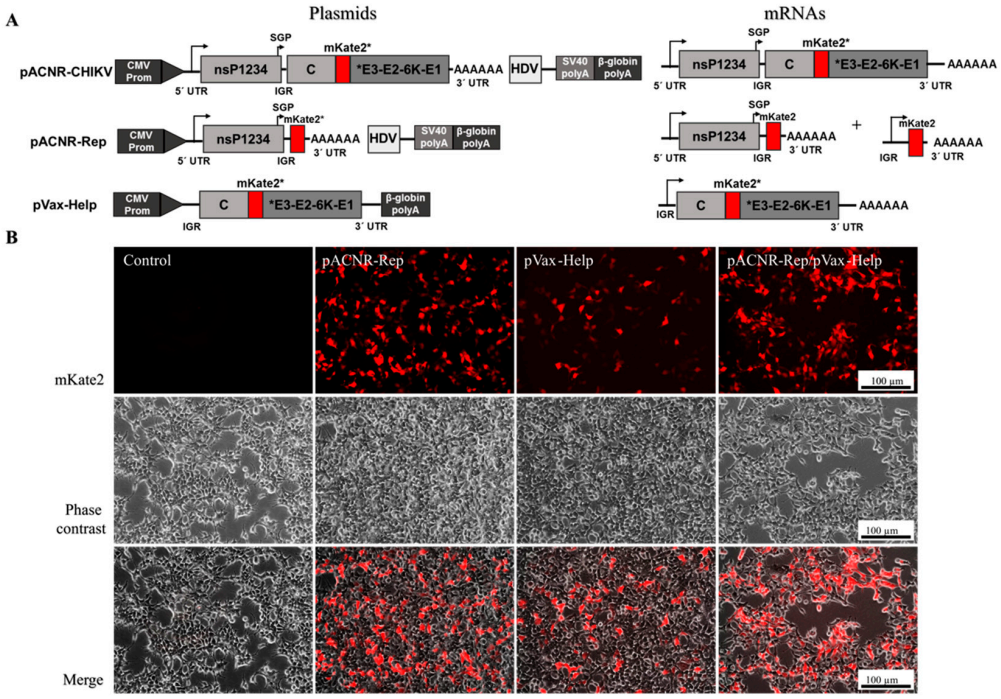

Figure 4. Generation and evaluation of replicon and helper vector system. (**A**) Constructions of pACNR-CHIKV, pACNR-Rep, and pVax-Help plasmids. The genomic CHIKV was inserted into the pACNR1811 vector from which the human cytomegalovirus (CMV) immediate early enhancer and promoter sequences, the SV40 polyA, and hepatitis delta virus (HDV) to generate pACNR-CHIKV. The structural proteins were removed to generate pACNR-Rep and derived from pVax-CHIKV and the nonstructural proteins were removed to generate pVax-Help. The asterisk means that mKate2 is a reporter gene. (**B**) HEK-293T cells transfected with pACNR-Rep, pACNR-Help, and co-transfected with both vectors after 48 h. Fluorescence micrographs with mKate2 expression (mKate2 channel), the morphology of cells in phase contrast, and a merge. Bar, 100 μm.

To demonstrate the functionality of the replicon and helper vector system, the supernatants of the co-transfected producer cells were removed within 24 h, the cells were washed, and a fresh culture medium was added. Figure 5A shows that the supernatant of cells co-transfected with pACNR-Rep and pVax-Help results in the expression of the reporter gene mKate2 after 48 h. However, the yield of the infection is extremely low compared to the number of infected cells when using the full-infection clone. Finally, electron transmission microscopy studies were performed on ultrathin sections of cells

co-transfected with pACNR-Rep and pVax-Help. Figure 5B shows the presence of type-I cytopathic vacuoles that contain small vesicles in their interior. Figure 5C shows a representative micrograph showing virions budding from the producing cell, while Figure 5D shows virions released from the cell with an approximate diameter of 66 nm.

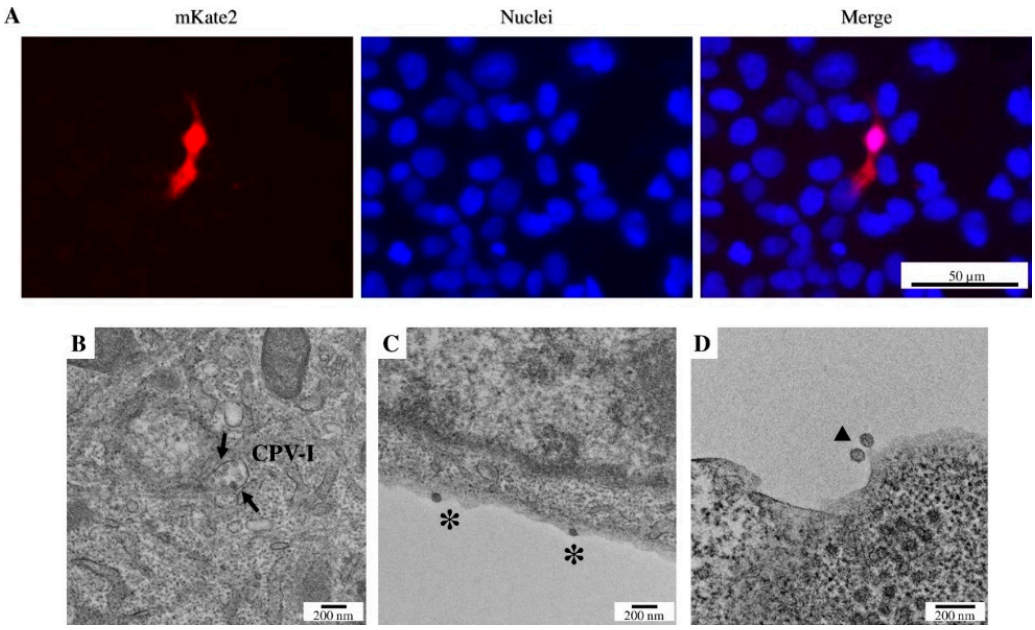

Figure 5. HEK-293T cells from the entry assay infected with the particles generated with the pACNR-Rep and pVax-Help system in (**A**) fluorescent-field mKate2 expression (Texas Red filter), nuclei stain (DAPI filter), and (**C**) overlap of mKate2 and nuclei micrographs, demonstrating that the particles generated by the co-transfection of both plasmids result in the expression of the gene of interest (mKate2) in the target cells. The bar indicates the same scale for all images, 50 µm. HEK-293T cells 48 h post-transfection with replicon and helper system were observed by transmission electron microscopy (TEM). (**B**) Cytopathic vacuole type 1 (arrow, CPV-I), (**C**) two virions budding from a membrane (*), and (**D**) two extracellular virions (▲). Bar B–D, 200 nm.

4. Discussion

The generation of infectious viral particles belonging to the *Togaviridae* family is usually achieved by electroporation of cultured cells with in vitro transcribed RNA [7,50,52–54]. Although this methodology is useful for the study of the biology of these viruses, it is not suitable for their use as viral vectors and/or for large-scale production of viral antigens (e.g., replication-deficient or inactivated viruses). The first aim of this project was to create an infectious clone of CHIKV in a CMV-driven plasmid for mammalian expression to eliminate the need for using in vitro transcripts. In our study, neither the CHIKV genome nor the replicon genome in the pVax plasmid results in the generation of the subgenomic RNA. This is consistent with the low levels of expression of another CMV-driven replicon where the viral polyA is not maintained intact [62]. The plasmid pVax-Helper resulted in the expression of the reported gene, which is consistent with the fact that the transcription of this mRNA does not depend on the CHIKV RdRp. The lack of any signal from the reporter gene in the cells transfected with pVax-CHIKV and pVax-Rep suggests that the β-globin polyadenylation termination signal introduces extra nucleotides that alter the structure of the 3′UTR region. This region is known to be essential for the RNA-dependent RNA polymerase (RdRp) to recognize the viral RNA [63]; mutations within this region could prevent the RdRp from recognizing the viral sequence and, thus, inhibit viral replication. To test this hypothesis, the 3′UTR was corrected by the addition of HDV ribozyme site after the viral polyA sequence and before the polyadenylation and termination signals. This approach has been used in previous studies with CHIKV, Kunjin, Venezuelan equine

encephalitis, and Sindbis virus because it excises the last nucleotide of the viral sequence from the first nucleotide of the ribozyme [46,54–56,64,65]. As expected and in line with the findings reported by Suzuki [56], the plasmids pACNR-CHIKV and pACNR-Rep expressed the mKate2 gene in transfected HEK-293T cells. Our results, along with those for CHIKV and other Alphaviruses, indicate that only the intact viral 3′UTR allows the RdRp to synthesize the subgenomic RNA from which the reporter gene is translated. Furthermore, these results along with those from Suzuki demonstrate that HEK-293T and Vero E6 cells are susceptible and permissible to CHIKV infection. The susceptibility of this cell line is most likely due to the presence of the cellular PHB1 in HEK-293T cells and TIM-1 in Vero E6 cells [30,66].

The presence of the reporter protein that translates in *cis* with the viral proteins allows us to directly evaluate, in real time, the replication of RNA and indirectly the translation of structural proteins. After 72 h.p.t., the integrity of the cell monolayer was lost, suggesting that the cells can no longer support viral replication, most likely due to cell death. Furthermore, at this time point, the media need to be replaced otherwise they becomes acidic, which, in turn, results in particles that are not infectious. This is consistent with the fact that CHIKV enters cells by endocytosis and, thus, it is possible that an acid medium changes the conformation of the E1 and E2 proteins of the extracellular particles to the one that is required for the virion to escape the endosome [67]. Further, we observed that a lower amount of DNA gives a higher yield of transfection. In our hands, this phenomenon occurs for almost any transfection with Lipofectamine 3000®; it is possible that high amounts of this reagent could be cytotoxic.

The infection in HEK-293T results in greater cell death than in Vero E6 cells. One possibility for this is that the latter does not have an interferon response. In fact, we observed the same effect with the Zika virus [60]. In addition, these particles generate, in Vero E6 cells, a cytopathic effect and plaques identical to those reported for this virus [68,69]. The particles observed by thin-section TEM near the plasma membrane have an approximate diameter of 50 to 60 nm, consistent with previous reports [70]. Although this variation has been previously observed [70], it is important to point out that we measured the diameters of the particles by thin-section TEM and not by negative-staining TEM of purified particles. Therefore, the virions that were not sectioned at the center of the particle will have a diameter smaller than expected. Recently, it was demonstrated that the mechanism of entry is clathrin-mediated in Vero cells (6 h.p.i.) [71]. Nonetheless, we were not able to observe the endocytosis of viral particles via thin-section TEM. This might be because the cells were fixed at 24 h.p.i., rather than 6 h.p.i. Furthermore, at 24 h.p.i. CHIKV, we were able to observe CPV-II and viral factory, supporting the idea that at this time, almost all the entry processes have already occurred and genome replication is taking place.

The generation of replicon and replicon/helper systems derived from Alphaviruses is not novel. However, for CHIKV, this system was generated by using plasmids for in vitro transcription rather than for mammalian expression [7,62,72,73]. Gläsker and co-workers generated a CHIKV replicon/helper system for transfection or electroporation with in vitro transcribed RNA rather than a CMV-driven plasmid [7]. However, this is not the only difference between the system presented here and the one from Gläsker. They generated the structural proteins by using two helper RNAs, while we only needed one plasmid. Further, the reporter gene they used was Gaussia luciferase, which is secreted into the supernatant instead of remaining intracellularly, as in our case.

On the one hand, the use of Gaussia luciferase has the advantage that this reporter system allows for the detection of lower amounts of the reporter gene than with most intracellular fluorescent protein. On the other hand, because Gaussia luciferase is secreted, it does not allow one to determine either the number of infected cells or to identify which cells are infected. In other words, by using mKate2 instead of Gaussia luciferase, we can determine the percentage and identity of infected cells by fluorescence microscopy. Utt et al. further reported a replicon system [62]; however, to detect the activity of the RdRp, they have to transfect cells with either two RNAs or CMV-driven plasmids: one molecule code

for the RdRp, while the other contains a reporter gene flanked by the regulatory sequences of the CHIKV RdRp. Unlike the system of Utt, our CMV-driven replicon plasmid generated here has a *cis* reporter gene and, hence, we only need to use one plasmid rather than two. Furthermore, the CMV-driven plasmid from Utt is barely functional, most likely because they did not fix the 3′UTR.

The fact that the infection of HEK-293T cells with the supernatant of the co-transfected cells at 48 h.p.t. results in the expression of the reporter gene confirms that the single-round infection particles packaged the replicon RNA. The presence of type-I cytopathic vacuoles indicates that the cellular localization of the non-structural proteins is the same as when an infectious clone is used [71]. Altogether, these data show that cells doubly transfected with pACNR-Rep and pVax-Helper result in the production of particles that are identical to CHIKV and that contain (at least some of them) the replicon RNA. However, the efficiency of infection with the single-round infectious particles produced in the doubly transfected cells was low compared to the infectious clone. This could be explained by the following scenario: in the producer cells, all the single-round infectious particles that are released prior to the collection time can enter non-transfected cells (as these cells are susceptible to CHIKV). Nonetheless, unlike with the infectious particles, the entry of single-round infectious particles does not result in the production of viral particles. Gläsker evaluated an alternative to resolve this problem and designed a replicon and helper system vectors, in which the synthesis of the helper RNA depends on the CHIKV RdRp [7]. It is important to point out that the single-round infectious particles reported by Gläsker use Gaussia luciferase as a reporter gene instead of a cytosolic protein. As was previously discussed, the use of a reporter protein that is secreted into the media does not allow one to determine the infectivity of these particles. Hence, it is likely that the yield of production of these particles is considerably lower than for the infectious virus, as we determined here, but it cannot be determined by measuring the bioluminescence of Gaussia luciferase. Furthermore, because not all the transfected cells contain both the replicon and helper plasmids, there is a fraction of particles that does not contain the replicon RNA. Nevertheless, the low yield of single-round infectious particles could be solved by either using a producer cell line that is not susceptible to CHIKV (which, given the wide tropism of this virus, is complicated to find) or by generating a stable cell line that continuously expresses the structural proteins. In fact, we are developing a stably transfected cell line that expresses the CHIKV structural proteins. In this system, when single-round infectious particles enter a stably transfected cell that is producing the structural proteins, it will package the replicon RNA. Therefore, with this system, we will be able to mimic the infectious process, but only when the single-round infectious particles enter cells that are stably expressing the structural proteins. This system will allow us to enrich the media with infectious particles.

5. Conclusions

Our results demonstrate that the generation of CHIKV infectious particles or of a CHIKV-derived RNA from a plasmid where transcription is driven by a Pol-II promoter and terminator requires that at least the 3′UTR be identical, as in the gRNA. The produced viral particles resulted in the expected cytopathic effect, plaques, and cytopathic vesicles, indicating that this plasmid results in particles that can mimic the infection of the wild-type virus. Further, the co-transfection of a replicon-independent helper plasmid and a replicon plasmid results in the assembly of single-round infectious particles that express the reporter gene in the target cells. However, this system has a low yield compared to the infectious clone.

The experiments described here are of great virological, biomedical, and biotechnological relevance. The pACNR-CHIKV plasmids can be used to produce infectious particles to study the virology of Alphaviruses, to determine the neutralizing of vaccine candidates, or to find antivirals that inhibit the CHIKV infectious cycle. The pACNR-Rep plasmid could be used in high-throughput approaches to find small molecules that inhibit the RdRp. The pVax-Helper plasmid could be used to understand the assembly process of CHIKV.

Finally, this replicon/helper system will allow us to perform studies to understand the viral infectious cycle in a manner where we can decouple genome replication and assembly.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/v15010132/s1, Table S1: Primers used for cloning, Figure S1: Fluorescent micrograph of cells transfected con pVax-Rep and pVax-Help, Figure S2: Virus diameter distribution; Figure S3: VLPs diameter distribution.

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Conflicts of Interest: The authors declare no conflict of interest.

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ANNEX

INDEX OF ADDITIONAL PRODUCTS

1. The Role of Virus-Like Particles in Medical Biotechnology. Mauricio Comas-Garcia, Mayra Colunga-Saucedo, and Sergio Rosales-Mendoza. Molecular Pharmaceutics 2020 17 (12), 4407-4420. DOI: 10.1021/acs.molpharmaceut.0c00828.

2. Comas-Garcia, M., Rubio-Hernández, E. I., Lara-Hernández, I., Colunga-Saucedo, M., Castillo, C. G., Comas-Garcia, A., Monsivais-Urenda,A & Zandi, R. (2022). Basic virological aspects of SARS-CoV-2. In Biomedical Innovations to Combat COVID-19 (pp. 1-30). Academic Press.

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Review

The Role of Virus-Like Particles in Medical Biotechnology

Mauricio Comas-Garcia,* Mayra Colunga-Saucedo, and Sergio Rosales-Mendoza*

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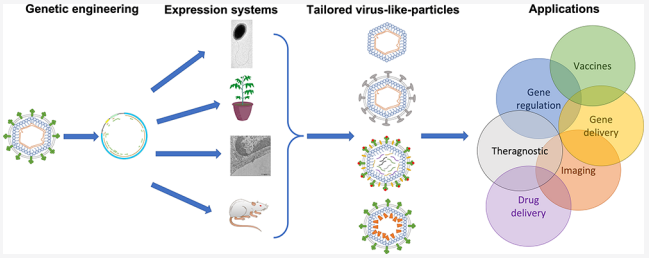
ABSTRACT: Virus-like particles (VLPs) are protein-based, nanoscale, self-assembling, cage architectures, which have relevant applications in biomedicine. They can be used for the development of vaccines, imaging approaches, drug and gene therapy delivery systems, and *in vitro* diagnostic methods. Today, three relevant viruses are targeted using VLP-based recombinant vaccines. VLP-based drug delivery, nanoreactors for therapy, and imaging systems are approaches under development with promising outcomes. Several VLP-based vaccines are under clinical evaluation. Herein, an updated view on the VLP-based biomedical applications is provided; advanced methods for the production, functionalization, and drug loading of VLPs are described, and perspectives for the field are identified.



KEYWORDS: *self-assembly, vaccine, imaging, chimeric antigen, gene therapy, drug delivery*

1. INTRODUCTION

The generation of virus-like particles (VLPs) was first described in 1955 with the *in vitro* reconstitution of the tobacco mosaic virus (TMV).[1] Since then, VLPs became key entities to study the physical properties and interactions that control virus assembly, which has set the basis for the development of bionanomaterials,[2,3] cargo/drug delivery systems,[4−8] nanoreactors,[9−12] gene therapy vehicles,[13−15] selective carriers for contrast media,[16−18] diagnostics,[19] and vaccines.[20−27] The development of different types of VLPs has allowed us to understand the rules of capsid assembly and genome packaging and to apply them in the rational design of self-replicating VLPs and/or nanomaterials that have functions that can be tailored to specific needs. The advent of genetic engineering and the advances in structural virology led to significant progress in the implementation of systems for the design and production of VLPs. These advances have allowed the assembly of VLPs structurally more complex than TMV. Moreover, the *in vitro* assembly of VLPs by using pure recombinant proteins still has key applications; however, the implementation of *in vivo* assembly systems opened, without a doubt, a path for new production processes and advanced VLPs applications in several areas. In fact, a big breakthrough in this field was the assembly of VLPs in bacteria, plants, insect cells, and mammalian cells. While we take it for granted, the fact that an animal virus can be assembled in plants,[28−31] yeast,[32,33] or *E. coli*[34−36] is a remarkable feat of molecular engineering and virology. The most advanced systems for the assembly of complex VLPs, typically from a virus that infects mammals, are based on mammalian and insect cell cultures. However, during the last decades, a remarkable achievement was the assembly in plants of VLPs resembling enveloped viruses (i.e., influenza virus). This type of platform offers not only a low production cost but also meets the biosynthesis requirements (access to a secretory route and particular glycosylation patterns). However, the greatest breakthrough will come with the achievement of attractive recombinant platforms, such as those based on plants and algae, for the assembly of enveloped VLPs having the same protein composition and 3D structure as in the wild-type virus assembled in the original host. As we will discuss later on, such improvement will allow us to generate particles with immunogenic properties or cell-recognition specificity similar to the wild-type virus but using a safer and much more economical expression system than mammalian/insect cell cultures. This last point becomes especially important when thinking of the scale required for the production of VLP-based vaccines for pandemics, such as the current one caused by the SARS-CoV-2 virus.[37]

2. BASIC CONCEPTS FOR THE DESIGN OF VIRUS-LIKE PARTICLES

One useful way to categorize VLPs is by classifying them as filled or empty, that is, if they package genomic material or

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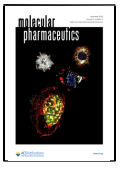

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Chapter 1

Basic virological aspects of SARS-CoV-2

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1.1 Introduction

Coronaviruses (CoVs) belong to a group of positive-sense single-stranded RNA viruses that have the largest genome known for a virus and use RNA as its genetic material. These viruses can infect a wide variety of animals, causing very different diseases that include common cold (humans), peritonitis (cats), hepatitis (mouse), and life-threatening pneumonia (humans). In fact, before the year 2002, most of CoV literature focused on viruses of veterinarian interest. This changed in 2002 and 2012 with the appearance of two novel human CoVs that cause Severe Acute Respiratory Syndromes, SARS-CoV and MERS-CoV, respectively. The localized epidemics caused by these two viruses served as warning events on how zoonotic transmission of CoVs between bats (or camels) and humans could result in the formation of new viruses. Unfortunately, the research on these viruses mostly caught only the attention of the groups that either previously worked with CoVs of veterinarian interest or lived in the regions affected by SARS-CoV and MERS-CoV.

The emergence of a novel human CoV (SARS-CoV-2) in Wuhan, China at the end of 2019 changed the world. By the first trimester of 2020, this virus was almost in every continent, causing an unprecedented halt of social, academic, economic, cultural, sports, and even political activities. This pandemic not only changed the way we behave but also the way science is done. Since the release of the first full-length SARS-CoV-2 genome, in February of 2020 by the group of Prof. Zhang (Wu et al., 2020), research groups from all around the world decided to learn about CoVs and develop new research interests. Most importantly, in order to fight this pandemic, we need to develop new biomedical and biotechnological tools to understand, at least at a basic level, how CoVs (especially SARS-CoV-2) function.

The goal of this chapter is to establish the minimal virological basis required to understand the viral infectious cycle. It is not our intention to provide a detailed review of every single aspect of the viral infectious cycle but to highlight key parts of it. In fact, we apologize to everyone whose work has not been highlighted. This chapter is intended to help the reader navigate through the rest of the book and to appreciate the novel biotechnological approaches that have been developed to fight the COVID-19 pandemic. This chapter presents the different steps of the viral infectious cycle as it occurs in the cell. First, we introduce the virus by giving a description of the genome organization and the function of some of its genes; afterward, we address virion entry, replication, assembly, and egress. Finally, we added a section regarding the immune response to a viral infection. The goal of this section is to provide the readers with the minimal knowledge required to understand and appreciate the rest of the chapters.

1.2 Genome organization and function

CoVs belong to the order *Nidovirales*, family *Coronaviridae*, and subfamily *Orthocoronavirinae*, which are further divided into four genera: Alphacoronavirus, Betacoronavirus, Gammacoronavirus, and Deltacoronavirus (V'kovski et al., 2020).

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Chapter 10

Virus-like particle-based vaccines against SARS-CoV-2

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10.1 Introduction

A few months after SARS-CoV-2 began spreading around the world, the World Health Organization (WHO) declared a Public Health Emergency of International Importance (WHO, 2020), and on March 11, 2020, a pandemic status was announced. This was the first pandemic status ever associated with a virus of the *Coronaviridae* family (Wu et al., 2020). According to the WHO, the number of confirmed cases of COVID-19 globally in late January 2021 was about 95 million and caused over 2 million deaths in 224 countries/regions, with the United States, India, Brazil, and Mexico having the highest number of cumulative cases (WHO Coronavirus Disease (COVID-19) Dashboard, 2020). The main clinical manifestations associated with this disease are fever, cough, dyspnea, myalgia, sore throat, and headache; however, the disease can progress to pneumonia, acute respiratory distress syndrome, and septic shock that can lead to death (Sakr et al., 2020; Singhal, 2020).

Given the unimaginable impact of the COVID-19 pandemic, the accelerated development efforts had led to some vaccines and monoclonal antibodies available in the market. Nonetheless, the efforts to find novel therapeutic and prophylactic treatments against COVID-19 should be continued, even now in the presence of licensed vaccines, to contain the spread of this disease. It has been shown that Remdesivir has broad antiviral activity (including SARS-CoV and MERS-CoV); therefore it has been proposed as a promising candidate against SARS-CoV-2. Monoclonal antibodies have also been developed and evaluated, and convalescent plasma therapy has been implemented as a prophylactic tool (Jean et al., 2020). However, the most effective method for the prevention and control of infectious diseases is vaccination; therefore the development of vaccines has been one of the goals to control the COVD-19 pandemic (Rosales-Mendoza et al., 2020)

The efforts made to develop vaccines against SARS-CoV and MERS have served as basis for the design of vaccine candidates against SARS-CoV-2. However, these efforts did not start from zero: SARS-CoV and SARS-CoV-2 share 79% of their genome (Kaur & Gupta, 2020); this similarity has been used as guideline for focusing the development of vaccines. These approaches use the S protein, or a region of it, as immunogen (Dong et al., 2020). Besides antigen election and formulations with carriers/adjuvants, the choice of an adequate vaccine production platform is fundamental to guarantee its scalability. Currently, vaccinology offers several vaccine types such as those based on live attenuated microorganisms, inactivated microorganisms, toxoids, recombinant proteins, nucleic acids, conjugated (targeting polysaccharides), and virus-like particles (VLPs).

The global picture of the SARS-CoV-2 vaccines, as of January 2021, includes 237 vaccine candidates; 173 of them are in the preclinical stage and 64 in the clinical stage (DRAFT landscape of COVID-19 Candidate Vaccine, 2020).

†. These authors contributed equally to this work.

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PLOS ONE

RESEARCH ARTICLE

Astrocytes derived from neural progenitor cells are susceptible to Zika virus infection

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Abstract

Zika virus (ZIKV) was first isolated in 1947. From its isolation until 2007, symptoms of ZIKV-caused disease were limited (e.g., fever, hives, and headache); however, during the epidemic in Brazil in 2014, ZIKV infection caused Guillain-Barré syndrome in adults and microcephaly in fetuses and infants of women infected during pregnancy. The neurovirulence of ZIKV has been studied using neural progenitor cells (NPCs), brain organoids, neurons, and astrocytes. NPCs and astrocytes appear to be the most susceptible cells of the Central Nervous System to ZIKV infection. In this work, we aimed to develop a culture of astrocytes derived from a human NPC cell line. We analyze how ZIKV affects human astrocytes and demonstrate that 1) ZIKV infection reduces cell viability, increases the production of Reactive Oxygen Species (ROS), and results in high viral titers; 2) there are changes in the expression of genes that facilitate the entry of the virus into the cells; 3) there are changes in the expression of genes involved in the homeostasis of the glutamatergic system; and 4) there are ultrastructural changes in mitochondria and lipid droplets associated with production of virions. Our findings reveal new evidence of how ZIKV compromises astrocytic functionality, which may help understand the pathophysiology of ZIKV-associated congenital disease.

Introduction

Zika virus (ZIKV) is a positive-sense single-stranded RNA [(+)ssRNA] virus that belongs to the *Flaviviridae* family and *Flavivirus* genus [1, 2]. ZIKV was first isolated, in the Zika forest in Uganda in 1947 from a *Rhesus* macaque that developed a febrile illness [3]. Since its isolation and until 2007, only a dozen cases were reported world-wide. However, in 2007, 2013, and 2014 large outbreaks occurred on Yap Island (Micronesia), French Polynesia, and Brazil, respectively. Disease symptoms were similar to those caused by other arboviruses such as dengue virus (DENV), including fever, rash, and headache [4–6]. However, during the epidemic

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"Construcción de plásmidos del virus de Chikungunya, de replicación y de ayuda para la transfección de células de mamífero."

TESIS DE DOCTORADO QUE PRESENTA

M. C. MAYRA COLUNGA SAUCEDO

DIRECTOR DE TESIS
DR. MAURICIO COMAS GARCÍA

ABRIL 2023

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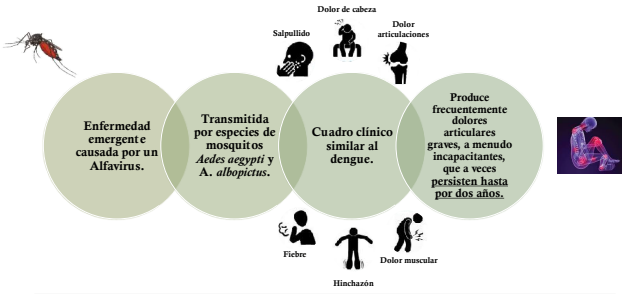
2

Fiebre Chikungunya — INTRODUCCIÓN

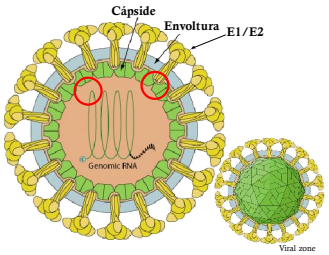


Schwartz, O., Albert, M. Biology and pathogenesis of chikungunya virus. Nat Rev Microbiol 8, 491–500 (2010).

3

Virus Chikungunya

- Familia *Togaviridae*
- Envuelto
- 240 monómeros de la proteína de la cápside
- Icosaédrico
- Diámetro 65-70 nm

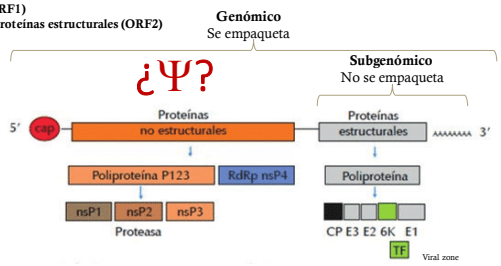


Schwartz, O., Albert, M. Biology and pathogenesis of chikungunya virus. Nat Rev Microbiol 8, 491–500 (2010).

4

Genoma del virus del Chikungunya

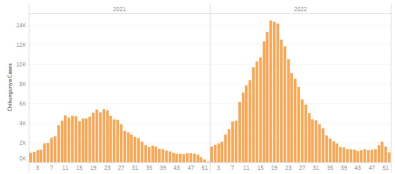
- ssRNA sentido positivo
- Genoma ≈12 kb
- 4 proteínas no estructurales (ORF1)
- 5 proteínas estructurales (ORF2)



Schwartz, O., Albert, M. Biology and pathogenesis of chikungunya virus. Nat Rev Microbiol 8, 491–500 (2010).

5

Casos de Chikungunya por semana epidemiológica (SE). Región de las Américas. 2021-2022

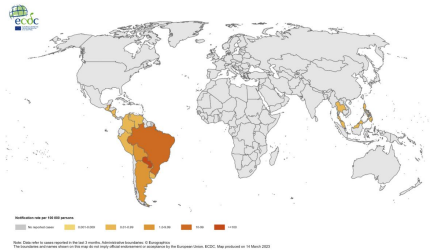


- 273 685 casos en 14 de los 52 países
- 50.6% confirmados
- Incidencia acumulada 2022 27.55 casos por 100 000 hab.
- Tasa de crecimiento de la curva 2021 fue 482%
- Tasa de crecimiento de la curva 2022 fue 813%
- Brasil 96.8% casos

Fuente: Datos ingresados en la Plataforma de Información de Salud para Américas (PLISA, PAHO / WHO) por los Ministerios e Institutos de Salud de los países y territorios de la Región. Disponible en: https://www.paho.org/plisa

6

Distribución geográfica mundial de contagios de CHIKV reportados hasta el 14 de marzo de 2023



European Centre for Disease Prevention and Control. Recuperado el 26 marzo de 2023.

7

NO EXISTEN TERAPIAS ANTIVIRALES O VACUNAS APROBADAS

Antivirales

Vacuna

9

Candidatos de vacunas en desarrollo clínico

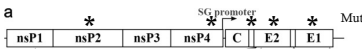
Vaccine strategy	Candidate name	Sponsor	Non-clinical development	Phase 1	Phase 2
Whole inactivated	–	USAMRIID and WRAIR	Immunogenicity and efficacy in mice and NHP	Completed	
	BBV87	Bharat Biotech International	Immunogenicity and efficacy in mice	CTRI/2017/02/007755	
Live attenuated	TSI-GSD-218 (181/clone 25)	Unites States Army Medical Research Institute of Infectious Diseases	Immunogenicity and efficacy in mice and NHP		Completed
	VLA1553	Valneva Austria GmbH	Immunogenicity and efficacy in mice and NHP	NCT03382964	
Viral vectored	MV-CHIK	Themis Bioscience GmbH	Immunogenicity and efficacy in NHP	EudraCT-2013-001084-23	NCT02961586 NCT03101111 NCT03228441 NCT03635086 NCT03907943
	ChAdOx1 Chik	Jenner Institute, University of Oxford	Immunogenicity and efficacy in mice	NCT03590392	
Virus like particles	VRC-CHKVLP059-00-VP PXVX0317 (former name: VRC-CHKVLP059-00-VP)	NIAID Emergent BioSolutions	Immunogenicity and efficacy in mice and NHP	NCT01489358 NCT03228441	NCT02562482 NCT03483961
mRNA	VAL-181388	Moderna Therapeutics	Immunogenicity and efficacy in mice; immunogenicity in NHP	NCT03325075	

Schrauf, S., et al. (2020). Current Efforts in the Development of Vaccines for the Prevention of Zika and Chikungunya Virus Infections. Frontiers in immunology, 11, 592. h

10

Vacuna de una cepa atenuada 181/clone25

Cepa 15561
Tailandia
Año 1962



Mutaciones sinónimas

taciones no sinónimas

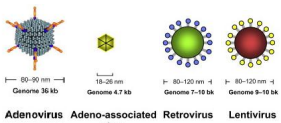
Fase I y Fase II
8% artralgias

Levitt, N., et al. (1986). Gorchakov R., et al. (2012).

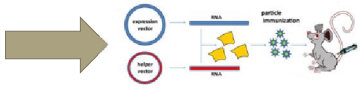
11

INTRODUCCIÓN

Vectores virales



Adenovirus | Adeno-associated virus | Retrovirus | Lentivirus



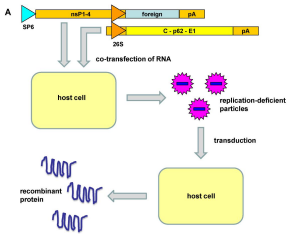
ARN auto replicante

Stefan Worgall, Ronald G. Crystal, Chapter 34 – Gene Therapy, Principles of Tissue Engineering (Fourth Edition), Academic Press, 2014, Pages 657-686.

12

INTRODUCCIÓN

Transcripción de genes *in vitro*



Genoma Alfavirus

- SP6
- T7
- CMV
- SFV
- CHIKV
- VEEV

Desventaja

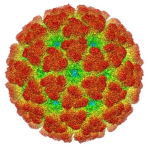
- Co-transfección
- Co-electroporación

Ballesteros-Briones, M.C. et al. (2020). Perri, S., et al. (2003). Bloom, K. , et al. (2021). Erasmus, J.H et al. (2020). Lundstrom, K. (2015).

13

JUSTIFICACIÓN

Es probable que el CHIKV se vuelva endémico del continente Americano, por lo tanto, la generación de vectores virales de CHIKV es fundamental para entender la biología del virus, el efecto del ciclo infeccioso en el hospedero, y así como para la generación de vacunas y antivirales.



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HIPÓTESIS

El inserción del total o parcial del genoma del CHIKV en un plásmido para expresión en células de mamíferos bajo el promotor CMV resultará en la expresión de partículas infecciosas y no infecciosas, respectivamente.

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OBJETIVO GENERAL

Clonar y caracterizar un sistema de plásmidos para la expresión en células de mamífero que resulten en vectores de replicación/de ayuda a partir de un clon del virus de Chikungunya.

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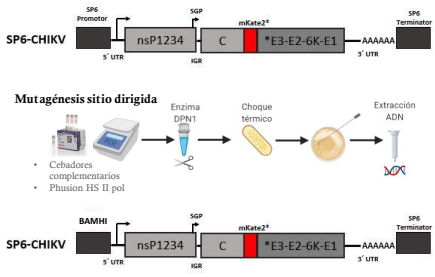
OBJETIVOS ESPECÍFICOS

1. Clonar un plásmido que contenga el genoma completo del virus de Chikungunya para expresar proteínas de interés en células de mamífero bajo un promotor eucarionte.
2. Construir un vector de replicación que contenga las proteínas no estructurales del virus de Chikungunya.
3. Construir un vector de ayuda que contenga las proteínas estructurales del virus de Chikungunya.
4. Transfectar células HEK-293T con el sistema de vectores de replicación y de ayuda generado.
5. Caracterizar la generación de viriones, del ARN auto replicante y de las proteínas estructurales mediante microscopia de fluorescencia y electrónica de transmisión en las células transfectadas.

17

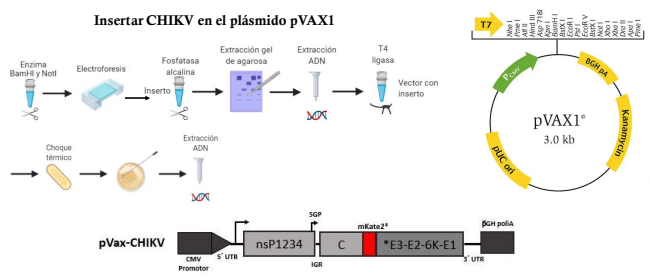
Objetivo 1) Clonar un plásmido que contenga el genoma completo del virus de Chikungunya para expresar proteínas de interés en células de mamífero bajo un promotor eucarionte.



Secuencias confirmadas

18

Objetivo 1) Clonar un plásmido que contenga el genoma completo del virus de Chikungunya para expresar proteínas de interés en células de mamífero bajo un promotor eucarionte.



Secuencias confirmadas

19

RESULTADOS

Alineamiento NCBI

Secuencias confirmadas

LANBAMA IPICYT

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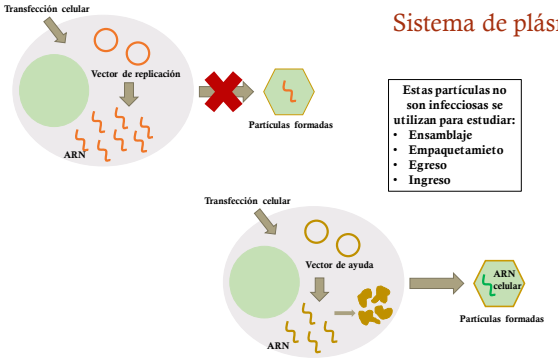
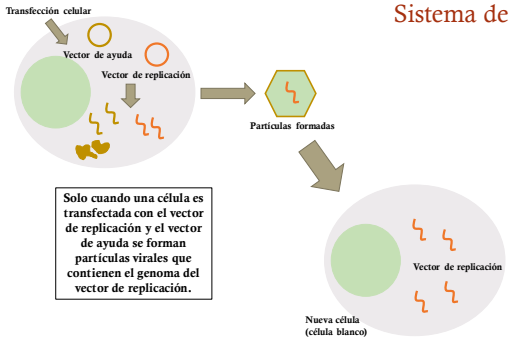
Sistema de plásmidos



21

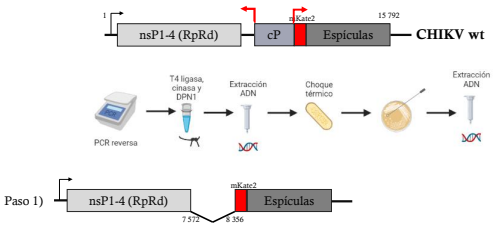
Sistema de replicones



Solo cuando una célula es transfectada con el vector de replicación y el vector de ayuda se forman partículas virales que contienen el genoma del vector de replicación.

22

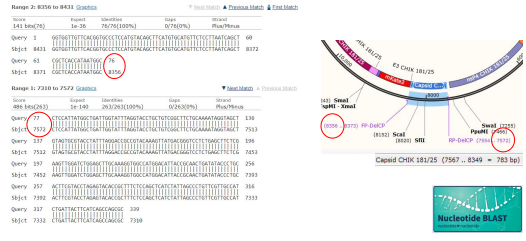
Objetivo 2) Construir un vector de replicación que contenga las proteínas no estructurales del virus de Chikungunya.



23

Objetivo 2) Construir un vector de replicación que contenga las proteínas no estructurales del virus de Chikungunya.

Alineamiento NCBI

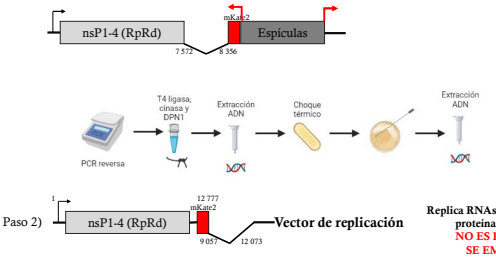


Secuencias confirmadas

LANBAMA IPICYT

24

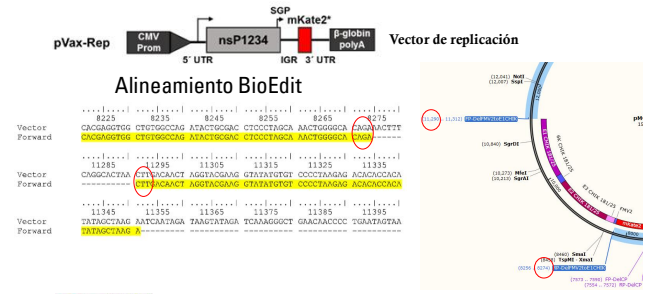
Objetivo 2) Construir un vector de replicación que contenga las proteínas no estructurales del virus de Chikungunya.



Replica RNAs virales pero no tiene proteínas estructurales.
NO ES INFECCIOSO.
SE EMPAQUETA.

25

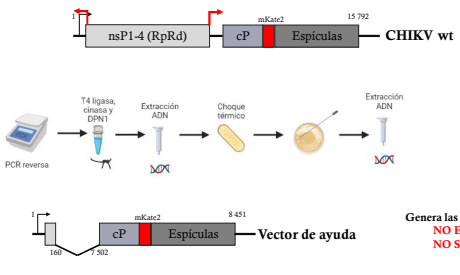
Objetivo 2) Construir un vector de replicación que contenga las proteínas no estructurales del virus de Chikungunya.



Secuencias confirmadas

26

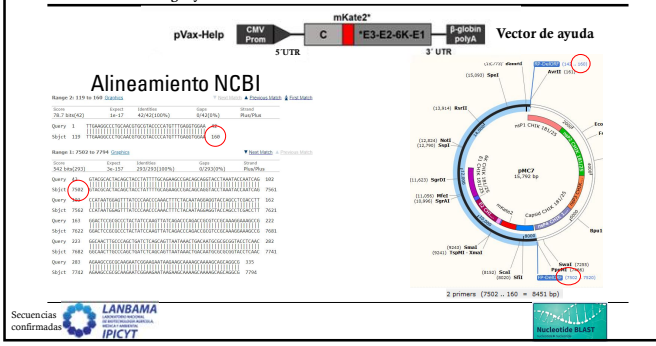
Objetivo 3) Construir un vector de ayuda que contenga las proteínas estructurales del virus de Chikungunya.



Secuencias confirmadas

27

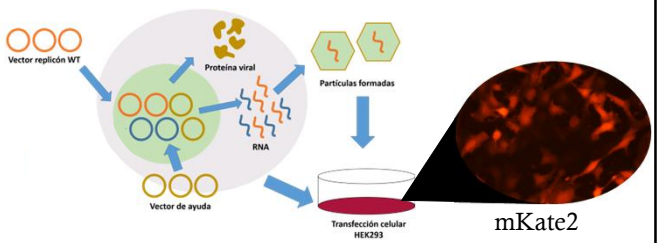
Objetivo 3) Construir un vector de ayuda que contenga las proteínas estructurales del virus de Chikungunya.



Secuencias confirmadas

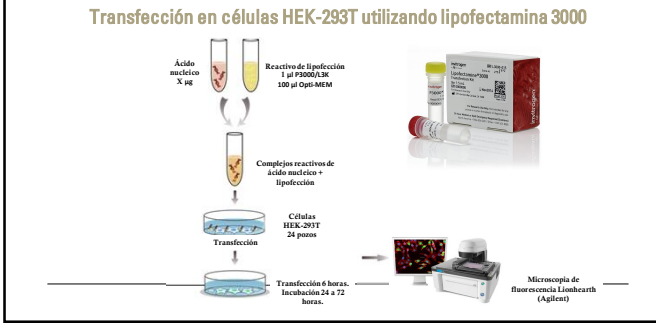
28

Objetivo 4) Transfectar las células HEK-293T con el sistema de vectores de replicación y de ayuda generado.



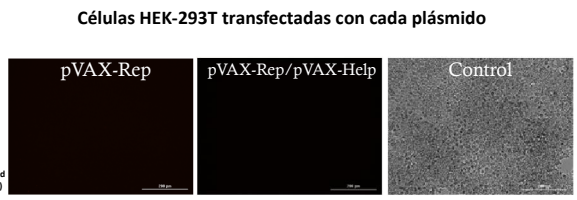
29

Objetivo 4) Transfectar las células HEK-293T con el sistema de vectores de replicación y de ayuda generado.



30

Objetivo 4) Transfectar las células HEK-293T con el sistema de vectores de replicación y de ayuda generado.



Colunga-Saucedo, M., et al. (2022).

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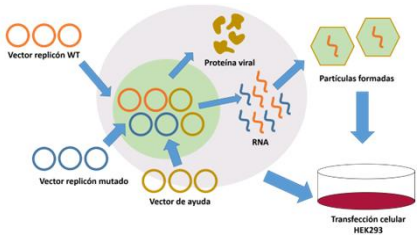
¿Baja eficiencia?

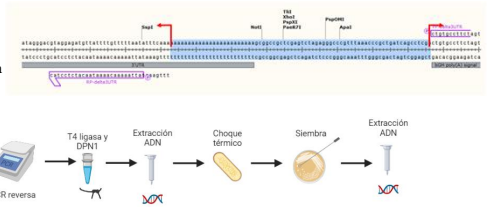
1. Replicación incorrecta y por lo tanto no generación del ARN sub genómico
2. Probabilidad de que la misma célula sea transfectada con ambos plásmidos



32

Estrategia 1) Modificación en la región 3´UTR

Vector de ayuda y Vector de replicación

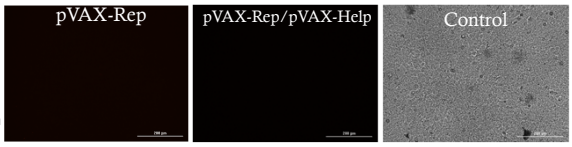


Secuencias confirmadas

33

Objetivo 4) Transfectar las células HEK-293T con el sistema de vectores de replicación y de ayuda generado.

Células HEK-293T transfectadas con cada plásmido



Colunga-Saucedo, M., et al. (2022).

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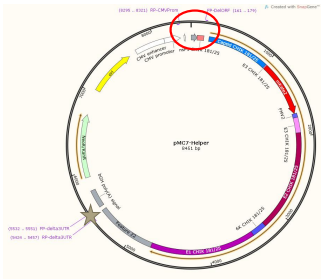
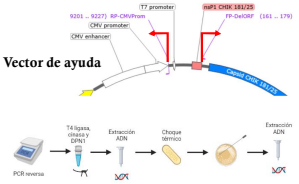
Estrategia 2) Modificación en la región 3´UTR y 5´UTR



Vector de ayuda

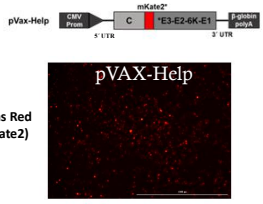


Secuencias confirmadas

35

Objetivo 4) Transfectar las células HEK293T con el sistema de vectores de replicación y de ayuda generado.

Células HEK-293T transfectadas con 1 µg plásmido



(b)

Colunga-Saucedo, M., et al. (2022).

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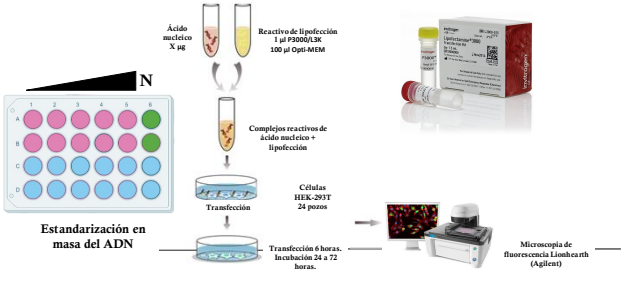
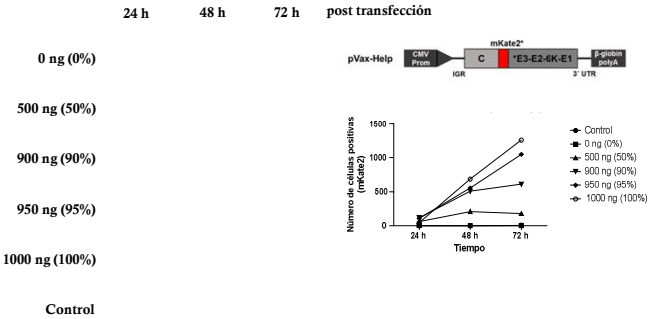
Objetivo 4) Transfectar las células HEK293T con el sistema de vectores de replicación y de ayuda generado.

Transfección en células HEK293T utilizando lipofectamina 3000



37

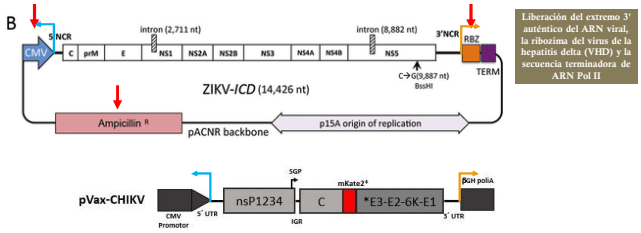
Células HEK-293T transfectadas con el vector de ayuda



38

Objetivo 1) Clonar un plásmido que contenga el genoma completo del virus de Chikungunya para expresar proteínas de interés en células de mamífero bajo un promotor eucariote.

Clonación del genoma del CHIKV en el vector pACNR1811



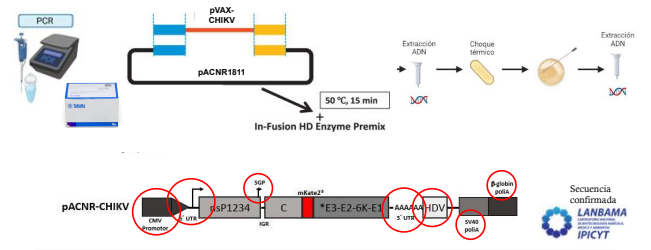
Liberación del extremo 3' auténtico del ARN viral, la ribozima del virus de la hepatitis delta (VHD) y la secuencia terminadora de ARN Pol II

Tsetsarkin KA, et al. (2016). Colunga-Saucedo, M., et al. (2022).

39

Objetivo 1) Clonar un plásmido que contenga el genoma completo del virus de Chikungunya para expresar proteínas de interés en células de mamífero bajo un promotor eucariote.

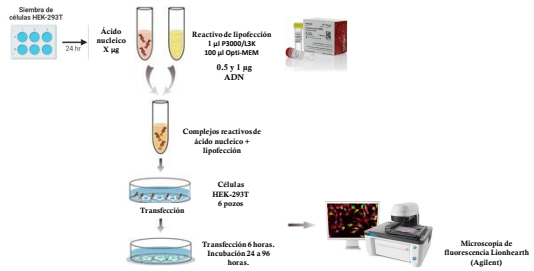
Clonación del genoma del CHIKV en el vector pACNR1811



Colunga-Saucedo, M., et al. (2022).

40

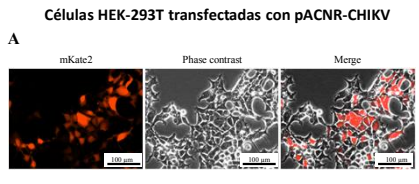
Transfección en células HEK-293T utilizando lipofectamina 3000



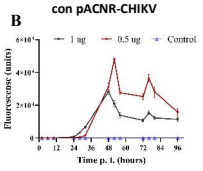
41

Objetivo 1) Clonar un plásmido que contenga el genoma completo del virus de Chikungunya para expresar proteínas de interés en células de mamífero bajo un promotor eucariote.

Células HEK-293T transfectadas con pACNR-CHIKV



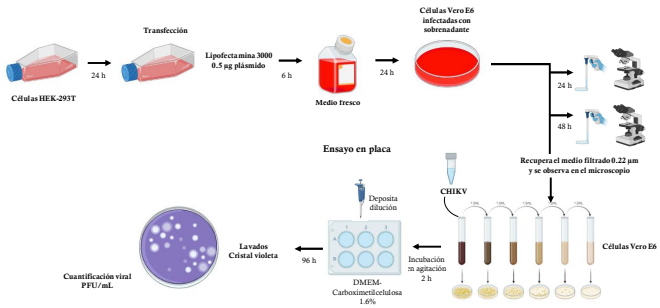
Cinética de la transfección de las células HEK-293T transfectadas con pACNR-CHIKV



Colunga-Saucedo, M., et al. Viruses (2022).

42

Objetivo 5) Caracterizar la generación de viriones, del ARN auto replicante y de las proteínas estructurales mediante microscopia de fluorescencia y electrónica de transmisión en células HEK-293T transfectadas.



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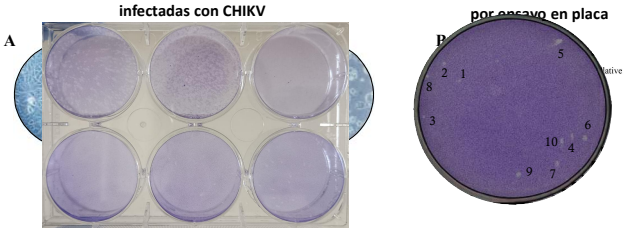
Objetivo 5) Caracterizar la generación de viriones, del ARN auto replicante y de las proteínas estructurales mediante microscopia de fluorescencia y electrónica de transmisión en células HEK-293T transfectadas.

Ensayo en placa de células Vero E6 infectadas con CHIKV

Cuantificación viral por ensayo en placa

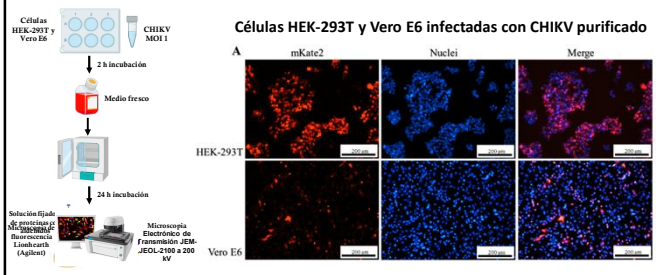


Colunga-Saucedo, M., et al. (2022). Rubio-Hernández EI, et al. (2023)

44

Objetivo 5) Caracterizar la generación de viriones, del ARN auto replicante y de las proteínas estructurales mediante microscopia de fluorescencia y electrónica de transmisión en células HEK-293T transfectadas.

Células HEK-293T y Vero E6 infectadas con CHIKV purificado



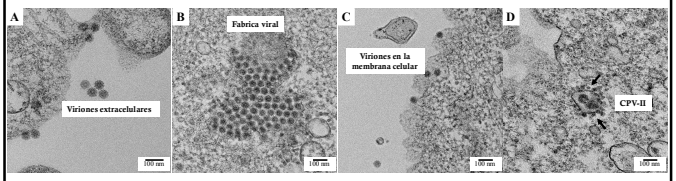
Colunga-Saucedo, M., et al. (2022).

45

Objetivo 5) Caracterizar la generación de viriones, del ARN auto replicante y de las proteínas estructurales mediante microscopia de fluorescencia y electrónica de transmisión en células HEK-293T transfectadas.

Tamaño y forma de las partículas virales Chikungunya en células HEK-293T infectadas

Diámetro ~50 nm

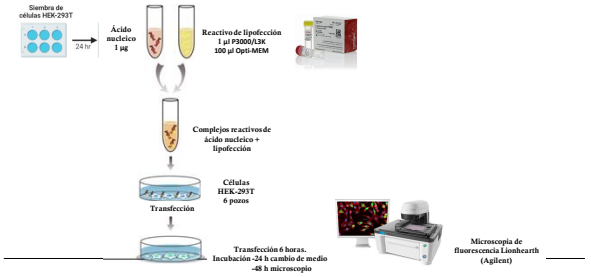


Biol. Miguel Angel Coronado-Ipiña, Laboratorio de Microscopia de Alta Resolución, CICSaB, UASLP.

Colunga-Saucedo, M., et al. (2022).

46

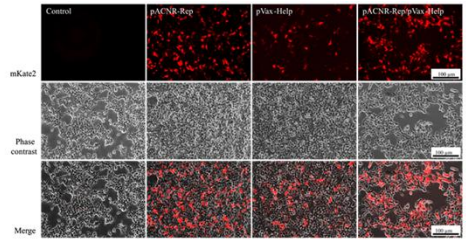
Transfección en células HEK-293T utilizando lipofectamina 3000 para la generación de los ARNs pACNR-Rep y pVax-Help



47

Objetivo 5) Caracterizar la generación de viriones, del ARN auto replicante y de las proteínas estructurales mediante microscopia de fluorescencia y electrónica de transmisión en células HEK-293T transfectadas.

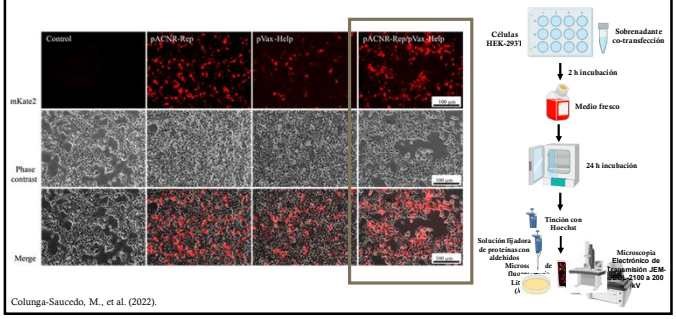
Células HEK-293T transfectadas con pACNR-Rep y pVax-Help



Colunga-Saucedo, M., et al. (2022).

48

Objetivo 5) Caracterizar la generación de viriones, del ARN auto replicante y de las proteínas estructurales mediante microscopia de fluorescencia y electrónica de transmisión en células HEK-293T transfectadas.

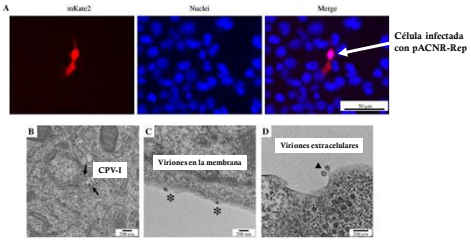


Colunga-Saucedo, M., et al. (2022).

49

Objetivo 5) Caracterizar la generación de viriones, del ARN auto replicante y de las proteínas estructurales mediante microscopia de fluorescencia y electrónica de transmisión en células HEK-293T transfectadas.

Células HEK-293T tratadas con el sobrenadante de células transfectadas con pACNR-Rep y pVax-Help

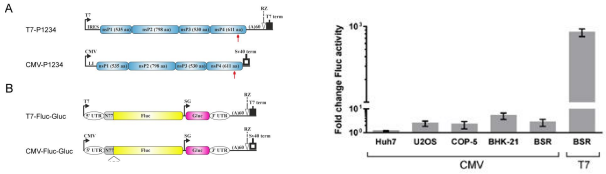


Colunga-Saucedo, M., et al. (2022).

50

DISCUSIÓN

Sistema de replicación *trans* del virus Chikungunya

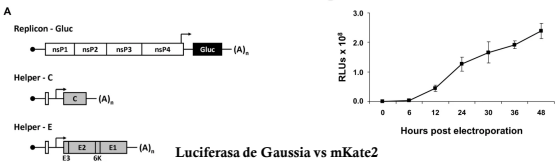


Utt, A., et al. (2016).

51

DISCUSIÓN

Ensayo de neutralización usando un vector de replicación de CHIK con luciferasa de Gaussia



Gläsker, S., et al. (2013).

Luciferasa de Gaussia vs mKate2

- **No hay cuantificación celular de infección y/o transfección**
- **Replicación *cis* del gen reportero**

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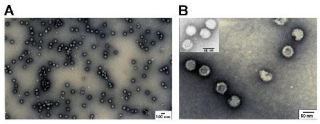
DISCUSIÓN

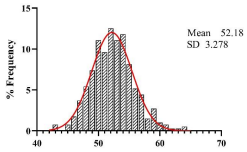
Partículas tipo virus Chikungunya



Noranate, N., et al. (2014).

- Vector de ayuda
- Células HEK-293
- ADN optimizado codones humano
- Tamaño 50 a 60 nm

Virus diameter distribution

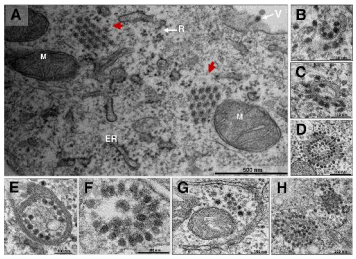


Colunga-Saucedo, M., et al. (2022).

53

DISCUSIÓN

Caracterización del ciclo de replicación del virus Chikungunya



Reis, E.V.S., et al. (2022)

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CONCLUSIONES

- La generación de partículas infecciosas de CHIKV o de un ARN derivado de CHIKV a partir de un plásmido donde la transcripción está bajo el control de un promotor y terminador eucarionte requiere que al menos la terminal 3'UTR sea idéntica a la del gARN.
- Las partículas virales producidas dieron como resultado el efecto citopático esperado; generaron placas y vesículas citopáticas. Esto que indica que la transfección resulta en una infección indistinguibles a la del virus silvestre.
- La co-transfección de un plásmido auxiliar independiente de replicón y un plásmido de replicón da como resultado el ensamblaje de partículas infecciosas de una sola ronda que expresan el gen reportero en las células diana.

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CONCLUSIONES

- El plásmido pACNR-CHIKV se pueden utilizar para producir partículas infecciosas para estudiar la virología de los alfavirus, para determinar la neutralización de vacunas candidatas o para encontrar antivirales que inhiban el ciclo infeccioso de CHIKV.
- El plásmido pACNR-Rep podría usarse en enfoques de alto rendimiento para encontrar pequeñas moléculas que inhiban la RdRp.
- El plásmido pVax-Help podría usarse para comprender el proceso de ensamblaje de CHIKV.

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PERSPECTIVAS

Debido a que este sistema tiene un bajo rendimiento en comparación con el clon infeccioso

- Generar un vector de ayuda dependiente de la RNA polimerasa viral t evaluar un sistema de plásmidos dependientes de la RNA polimerasa viral en células de mamífero.
- Generar una línea estable que exprese las proteínas estructurales.

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PERSPECTIVAS

Gracias a los plásmidos desarrollados en esta tesis se están realizando los siguientes proyectos:

1. Generación de una línea establemente transfectada con el vector de ayuda (Tesis de licenciatura).
2. Generación de mutaciones en las proteínas espículas para entender la mecánica de la gemación del CHIKV (Tesis de maestría).
3. Estudio sobre las interacciones entre las proteínas de la cápside que llevan al ensamblaje viral y la generación de viriones hiperestables (por someterse al Wellcome Trust).

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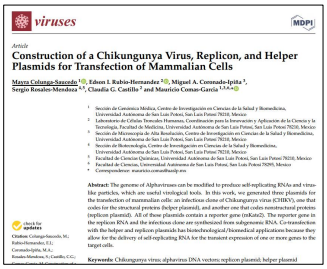
PUBLICACIONES


viruses

MDPI

Article

Construction of a Chikungunya Virus, Replicon, and Helper Plasmids for Transfection of Mammalian Cells

Mayra Colunga-Saucedo [1,†], Edson I. Rubio-Hernandez [2,†], Miguel A. Coronado-Ipiña [3], Sergio Rosales-Mendoza [4,*], Claudia G. Castillo [2] and Mauricio Comas-García [1,5,6,*]

Colunga-Saucedo, M.; Rubio-Hernandez, E.I.; Coronado-Ipiña, M.A.; Rosales-Mendoza, S.; Castillo, C.G.; Comas-Garcia, M. Construction of a Chikungunya Virus, Replicon, and Helper Plasmids for Transfection of Mammalian Cells. Viruses 2023, 15, 132. https://doi.org/10.3390/v15010132

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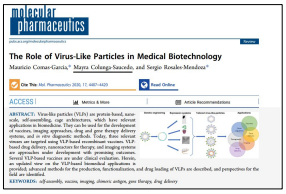
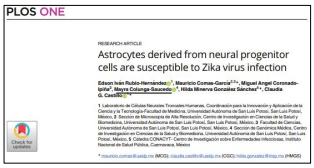
PUBLICACIONES


molecular pharmaceutics

The Role of Virus-Like Particles in Medical Biotechnology

Mauricio Comas-García,* Mayra Colunga-Saucedo, and Sergio Rosales-Mendoza*


PLOS ONE

RESEARCH ARTICLE

Astrocytes derived from neural progenitor cells are susceptible to Zika virus infection

Edson Iván Rubio-Hernández[1], Mauricio Comas-García[2,3]*, Miguel Angel Coronado-Ipiña[4], Mayra Colunga-Saucedo[2,3], Hilda Minerva González Sánchez[5,6], Claudia G. Castillo[2]*

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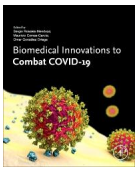
PUBLICACIONES



Chapter 1

Basic virological aspects of SARS-CoV-2

M. Comas-García[1,2,3], E.I. Rubio-Hernández[4], I. Lira-Hernández[4], M. Colunga-Saucedo[2], C.G. Castillo[4], A. Comas-García[5,6], A. Montvale-Urrieta[5,6] and R. Zandi[6]

Chapter 10

Virus-like particle-based vaccines against SARS-CoV-2

I. García-Silva[1,2,3], M. Colunga-Saucedo[2,3], C. Alvendarez-Rodríguez[2,3], C. Miranda-López[2,3], S. Rosales-Mendoza[2,3] and M. Comas-García[2,3]

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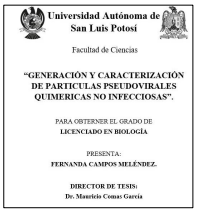
COLABORACIONES


Universidad Autónoma de San Luis Potosí

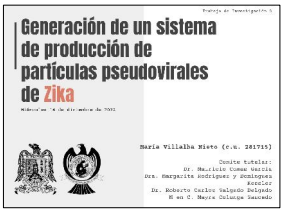
Facultad de Ciencias

"GENERACIÓN Y CARACTERIZACIÓN DE PARTICULAS PSEUDOVIRALES QUIMERICAS NO INFECCIOSAS".

PARA OBTENER EL GRADO DE
LICENCIADO EN BIOLOGÍA

PRESENTA:
FERNANDA CAMPOS MELÉNDEZ.

DIRECTOR DE TESIS:
Dr. Mauricio Comas Garcia


Generación de un sistema de producción de partículas pseudovirales de Zika

María Villalba Nieto (e.u. 281715)

Comité tutelar:
Dr. Mauricio Comas García
Dra. Margarita Rodríguez y Domínguez Rosales
Dr. Roberto Carlos Salgado Delgado
M en C. María Salazar Saucedo

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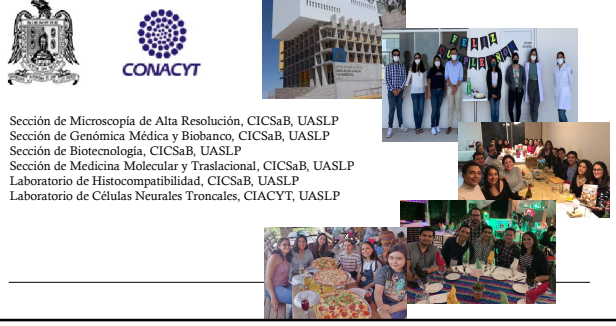
AGRADECIMIENTOS



1. Sección de Microscopía de Alta Resolución, CICSaB, UASLP
2. Sección de Genómica Médica y Biobanco, CICSaB, UASLP
3. Sección de Biotecnología, CICSaB, UASLP
4. Sección de Medicina Molecular y Traslacional, CICSaB, UASLP
5. Laboratorio de Histocompatibilidad, CICSaB, UASLP
6. Laboratorio de Células Neurales Troncales, CIACYT, UASLP

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