Chapter 2 Molecular biology: tools for the study of re-emerging diseases

Capítulo 2 Biología molecular: herramientas para el estudio de enfermedades reemergentes

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DOI: 10.35429/H.2022.6.1.13.25

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A. Marroquín, L. Castillo, S. Soto, L. Cruz. (Coord.) CIERMMI Women in Science TXIX Biological Sciences. Handbooks-©ECORFAN-México, Querétaro, 2022.

Abstract

Chikungunya fever is a disease caused by the chikungunya virus (CHIKV), which is transmitted by hematophagous female mosquitoes (Mohan, 2010). Chikungunya Fever is a re-emerging illness with a great global impact, causing severe public health problems (Mohan, 2010) (Gérardin, 2011) (Sebastian, 2009). CHIKV contains a single-stranded RNA genome (+) with two open reading frames (ORFs), one for nonstructural proteins and the other for structural proteins (Caglioti, 2013). Little is known about the specific role nonstructural proteins (NSPs) play during viral replication. Nevertheless, NSP1 is known to be involved in the induction of cytoskeleton and plasma membrane changes and cell prolongations such as filopodia (Laakkonen, 1998). Currently, there are no commercial antibodies to detect NSP1; hence, it is of utmost importance to design tools that would allow us to study the role of this protein during the viral cycle. In this study, primers for CHIKV NSP1 were designed and used to amplify cDNA, which was cloned into the pPROEX Htb expression vector. The optimal expression time for NSP1 was determined, and the expressed protein was purified. The recombinant NSP1 (rNSP1) protein obtained using this process was used to immunize rats to obtain polyclonal antibodies. These antibodies were tested in Vero cells infected with CHIKV and were observed using immunofluorescence assays, showing the recognition of viral proteins in their native form.

Chikungunya, NSP1, Antibody, Recombinant, Clonation, Purification

Resumen

La fiebre de Chikungunya es una enfermedad causada por el virus de Chikungunya (CHIKV), el cual es transmitido mediante la picadura de los moquitos hematófagos hembras (Mohan, 2010). La fiebre de Chikungunya es una enfermedad reemergente con un gran impacto global que ha causado severos problemas en salud pública (Mohan, 2010)(Gérardin, 2011) (Sebastian, 2009). CHIKV contiene una cadena monocatenaria de RNA y polaridad positiva. Este genoma tiene 2 marcos de lectura (ORFs) que codifican para dos poliproteínas: la no estructural y la estructural (Caglioti, 2013). Poco se conoce acerca de la actividad de las proteínas no estructurales (NSPs) durante la replicación del virus. Para NSP1, se ha observado que participa durante la formación de prolongaciones celulares (como los filopodios), así como en la remodelación del citoesqueleto y membranas plasmáticas (Laakkonen, 1998). Actualmente, en el mercado no hay anticuerpos específicos que detecten a NSP1, por lo que es de suma importancia el diseño de herramientas que nos permitan estudiar el papel de esta proteína durante el ciclo viral. En este estudio se diseñaron oligos para NSP1 y se emplearon para la amplificación del cDNA. Posteriormente este cDNA se clonó en el vector de expresión pPROEX Htb. La proteína recombinante NSP1 (rNSP1) fue purificada y utilizada para la inmunización de ratas. Los anticuerpos policlonales obtenidos fueron probados en células VERO infectadas con CHIKV y, mediante ensayos de inmunofluorescencia, se detectó a la proteína NSP1 en su forma nativa.

Chikungunya, NSP1, Anticuerpo, Recombinante, Clonación, Purificación

2.1 Introduction

CHIKV causes Chikungunya fever, which is considered a re-emerging illness and has a severe impact in public health. This is due to its debilitating and lasting sequelae, mainly of a rheumatological type, resulting in expensive treatments and therefore burdening the health sector of the countries in which they occur, such as Mexico, Argentina, Colombia and Bolivia, which have the most confirmed chikungunya cases (OMS, 2020).

Symptoms of CHIKV appear within 3-7 days of infection and most patients with acute infection show a fever higher than 38.9 °C, headache, rash, fatigue, nausea, vomiting, joint pain, and conjunctivitis. Almost all infected people develop severe polyarthralgia, more frequently in the wrists, elbows, fingers, knees and ankles, and it tends to weaken the patient. In some cases, joint pain, fatigue, and neuritis continue to be observed up to ten months after infection, with 43–75% of infected people reporting to have some late- or prolonged-onset symptoms (on average, two years after infection), such as recurrent joint pain or joint stiffness (Simon, 2007) (Gérardin, 2011) (Manimunda, 2010).

CHIKV belongs to the Togaviridae family and Alphavirus genus. Most alphaviruses are transmitted by mosquitoes to vertebrate hosts, such as humans, nonhuman primates, birds, amphibians, reptiles, rodents, and pigs (Chen, 2018). CHIKV has an icosahedral form with a diameter of 60-70 nm and is wrapped by a phospholipid layer. It contains a genome approximately 11.8 kb long, which is a single linear chain of positive RNA flanked by two UTRs (untranslated regions) at the 5′ end and a poly-A tail at the 3′ end (Caglioti, 2013). The CHIKV genome has two open reading frames (ORFs), the first of which encodes four nonstructural proteins (NSPs): NSP1, NSP2, NSP3, and NSP4. The second ORF encodes five structural proteins, including those that form the capsid (C) and the envelope glycoproteins E1, E2, E3, and 6k (Li, 2012).

Cell infection starts when CHIKV binds to the cell membrane, mainly through the interaction of the E2 protein. Next, the virus enters the cell via clathrin-dependent endocytosis. When an endosome is formed, the endosome pH begins to decrease, leading to a conformational rearrangement of the virus and E2-E1 heterodimer dissociation. E1 then participates as a fusogenic peptide, inducing the fusion of the endosome and the viral coat, allowing the RNA to be released into the cytosol. The nonstructural proteins are translated as polyproteins and a replicative complex (RC) is formed, wherein genomic RNA and other nonstructural proteins are cleaved to the plasma membrane where RNA synthesis occurs. The double-stranded RNA formed leads to the synthesis of genomic and subgenomic viral RNAs. Subgenomic RNA is translated to produce structural proteins that form the viral capsid, which interacts with genomic RNA, giving rise to new viruses, and leading to cell exit for the infection of new cells (Cunha, 2020).

Nonstructural protein 1 (NSP1) is highly involved in viral replication. This molecule is 535 amino acids long (~60 kDa) and has three domains: an N-terminal domain (NT) (with methyltransferase [MT] and guanylyltransferase functions), a membrane-binding domain (MB), and a C-terminal domain (CT). The NT domain is involved in the methylation and capping of viral RNA. The MB anchors the replication complex (CR) to the cell membrane. The function of C-terminal domain is not yet known (Kumar, 2018) (Cunha, 2020). Thus, NSP1 participates in binding the replication complex (RC) to the inner part of the plasma membrane and capping the genomic and subgenomic RNA (Ahola, 1995). Additionally, it is believed that NSP1 is involved in membrane reorganization during cell infection; however, many aspects of this phenomenon remain to be elucidated (Kumar,2018).

2.2 Methodology

2.2.1 Designing primers

Primers were designed to amplify the coding sequence for NSP1. The sequence of the complete CHIKV genome from the first clinical case in Mexico reported by InDRE (strain 51CHIK; GenBank: KP851709.1) was used as a template. Two primers were designed, which were approximately 20 to 25 base pairs in length and had an alignment temperature between 56 and 59 °C. The primers were then analyzed using NEBcutter V2.0 and Clustal Omega software to rule out other restriction sites of the enzymes to be used within the sequences and corroborate that their sequences were not complementary.

2.2.2 Viral RNA and cDNA preparation

A Vero cell culture was infected with Chikungunya virus at 5 MOI for 12 h. Subsequently, the cell monolayer was washed with Dulbecco's Modified Eagle Medium (DMEM) without Fetal Bovine Serum (FBS), and trizol reagent was added. The resulting solution was vigorously mixed at room temperature (20-25°C) for 5 min. Next, 200 µL of a cold solution of chloroform and isoamyl alcohol (49:1, v/v) was added, mixed via inversion, and centrifuged at 20,000 × g at 4°C for 15 min, and the aqueous phase was recovered. Next, 500 µL of cold isopropanol was added the aqueous phase, mixed via inversion, centrifuged at 20,000 × g for 10 min, and the supernatant was removed. The pellet was then washed with 75% ethanol. Finally, the isolated RNA was resuspended in DEPC water and quantified. A mixture containing dNTPs, DTT, 200 U/µL of the enzyme SuperScript III (reverse transcriptase), and the previously extracted RNA was added to specific primers previously designed to amplify the NSP1 sequence via RT-PCR.

The coding region for NSP1 was amplified using the designed primer pairs, and the synthesized cDNA was used as a template for further amplification. For this, 35 cycles were carried out as follows: initial denaturation at 95 °C for 5 min, primer annealing at 56.8 °C for 1 min, and cDNA extension at 72 °C for 7 min.

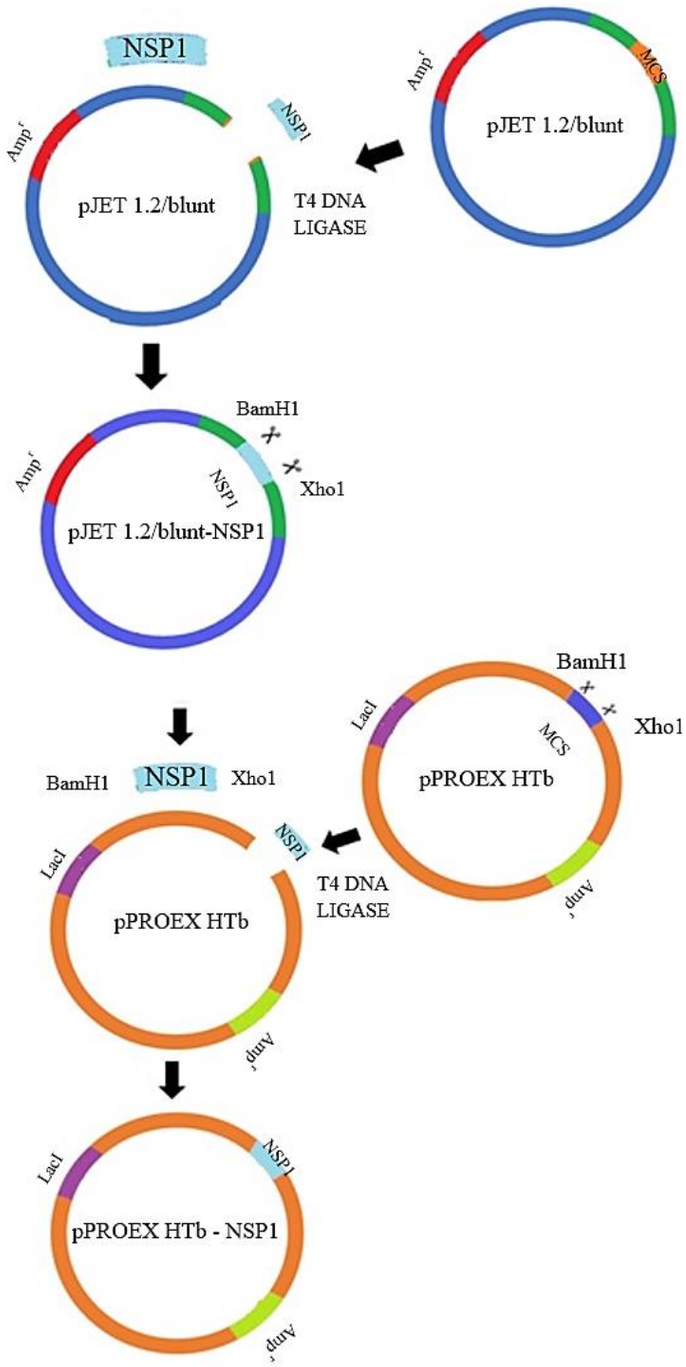
2.2.3 Construction design and obtaining recombinant *Escherichia coli* cells

The amplicon (NSP1) was ligated into a pJET transfer vector following the cloning protocol for blunt ends (CloneJET PCR Cloning kit, Thermo Scientific). Briefly, the PCR product was purified from the electrophoresis gel (QIA Quick Gel extraction kit) and digested to produce blunt ends. Nuclease-free water and the reaction buffer (Thermo Scientific, 1×) were mixed and incubated at 70 °C for 5 min. Subsequently, the vector and T4 DNA ligase (Thermo Scientific, 1×) were added and incubated at room temperature for 5 min. The above mixture was used to transform competent XL10 Gold E. coli cells, which were subject to heat shock at 42 °C for 45 s, placed on ice for 1 min, and 400 µL of LB medium was added. The transformed cells were then incubated at 37 °C for 1 h with constant agitation. Finally, the cells were spread onto plates with LB medium supplemented with 120 µg/mL ampicillin and incubated at 37 °C for 24 h to screen for positive transformants.

Next, the digestion of the expression vector pPROEX Htb was carried out using the restriction enzymes *BamHI* and *XhoI*. This was done for directed cloning during ligation, wherein the amplicon was inserted in the 5′-3′ direction, thus resulting in the appropriate reading frame.

The next step was the release of the NSP1 gene via digestion at 37 °C for 2 h with the enzymes *BamHI* and *XhoI* from the previous construct (NSP1-pJET) and transferring the gene into the expression vector pPROEX HTb. The transformation process was carried out using competent BL21 E. coli, as described previously for the XL10 Gold strain. General cloning strategy of NSP1 into the transfer pJET 1.2/ blunt and expression pPROEX HTb vectors is shown below (Figure 2.1).

Figure 2.1 Schematic representation of general cloning strategy for pJET1.2 and pPROEX Htb vectors and the final construction pPROEX HTb-NSP1 flanked by *BamHI* and *XhoI* restriction sites



2.2.4 Evaluating the Expression and purification of NSP1

To elucidate the optimal expression time of NSP1, induction kinetics were evaluated in recombinant E. coli BL21 cells incubated at 37 °C with agitation, with or without induction agent (IPTG, 100 mM). Bacterial aliquots were taken at 0, 3, 6, 9, and 12 h post-induction. Recombinant bacterial cultures were lysed and analyzed via western blotting using an antibody against His-tag. The proteins were exposed using a Super Signal West Femto Maximum Sensitivity Substrate chemiluminescence kit (Thermo Fisher Scientific) and were analyzed with a ChemiDoc system (Bio-Rad).

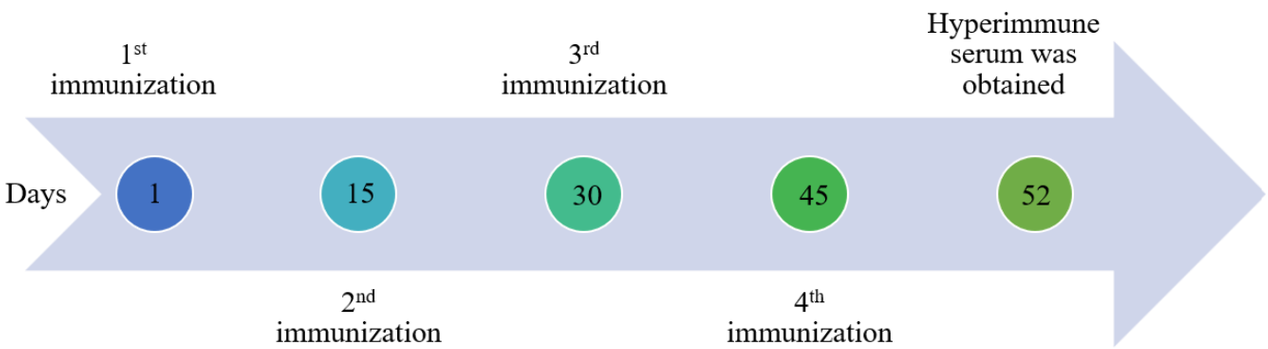
To purify NSP1, it was produced in 1 L bacterial culture for 9 h post-induction, as previously standardized. Bacteria were centrifuged at 4,000 × g, resuspended in lysis buffer, stirred at room temperature for 60 min, centrifuged at 10,000 × g at room temperature for 30 min, and the supernatant was recovered.

Meanwhile, a NiNTA Resin 3x (QIAGEN) was washed with B lysis buffer (The QIAexpressionist™, QIAGEN; 100 mM NaH2PO4, 10 mM Tris-Cl, and 8 M urea; pH 8.0) and 100 µL of resin per 100 mL of culture was added. The lysates were mixed with the resin and incubated for 1 h at room temperature with gentle stirring. The mixture was passed through a column and the eluate was recovered. Subsequently, the mixture was washed twice with 4 mL of B lysis buffer (pH 8.0), followed by two washes with 4 mL of C buffer (pH 6.3). Afterward, 2 mL of D buffer (pH 5.9) was added, and eluates were recovered in 500-µL aliquots. Finally, 2 mL of E buffer (pH 4.5) (The QIAexpressionist ™: QIAGEN) was added, and 500-µL eluates were recovered.

2.2.5 Wistar rat immunization protocol and evaluation of hyperimmune serum by immunofluorescence

Two Wistar rats were immunized intraperitoneally with four doses of 150 µg purified NSP1. Each dose was administered at 15-day intervals (Figure 2.2). The first immunization included recombinant NSP1 protein mixed with complete Freund's adjuvant. The next dose was administered with incomplete Freund's adjuvant. After immunization, bleeding was performed to obtain the serum.

Figure 2.2 NSP1 immunization protocol



2.2.6 Immunofluorescence assay

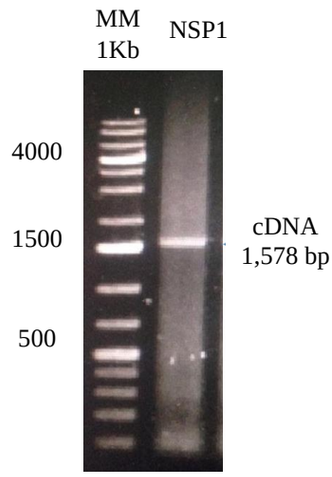
Approximately 8×10^5 Vero cells were seeded in each well of a 24-well plate and grown until confluence. The monolayers were infected with CHIKV at 5 MOI for 5 and 24 h. After the infection, the cells were fixed with 300 µL of 4% paraformaldehyde for 20 min and washed twice with sterile 1× PBS. Subsequently, the cells were permeabilized by adding 300 µL of 0.1% Triton X-100 in PBS + 0.2% gelatin and incubated with agitation for 20 min in the dark. The cells were then blocked with 300 µL of 10% goat serum (PBSTCH) diluted in 0.1% PBS-Triton X-100 and incubated with agitation for 1 h. After incubation, the slides were placed with 20 µL of primary antibody from the immunized rats, diluted 1:100 in PBSTCH, and incubated for 1 h at room temperature. The corresponding secondary antibody (anti-rat IgG-FitC, diluted 1:50 in PBSTCH) was added, and the cells were incubated for another 1 h at room temperature in the dark. Finally, the slides were washed with 0.1% PBS-Triton. The slides were observed under an epifluorescence microscope (Leica) at 60× magnification or in a confocal microscope (Leica) at 40× magnification.

2.3 Results and Discussion

2.3.1 Design and cloning of recombinant plasmids

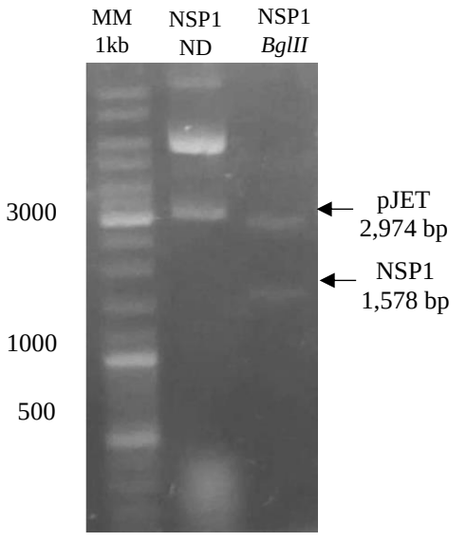
Two primers, 24 base pairs long each, were designed and flanked by the restriction sites of *XhoI* and *BamHI* in the forward and reverse directions, respectively, with an annealing temperature of 56.89 °C. After a series of amplification cycles, the gene encoding the NSP1 protein was obtained at an expected size of approximately 1,578 bp (Figure 2.3).

Figure 2.3 NSP1 RT-PCR electropherogram. The NSP1 sequence was amplified using the forward 5′-CGC-GGA-TCC-ATG-GAT-TCT-GTG-TAC-3′ and the reverse primer 5′-CTA-TCT-CGA-CCA-CGA-GAG-CTC-GAA-3′, which were flanked by the *BamHI* and *XhoI* restriction sites, respectively



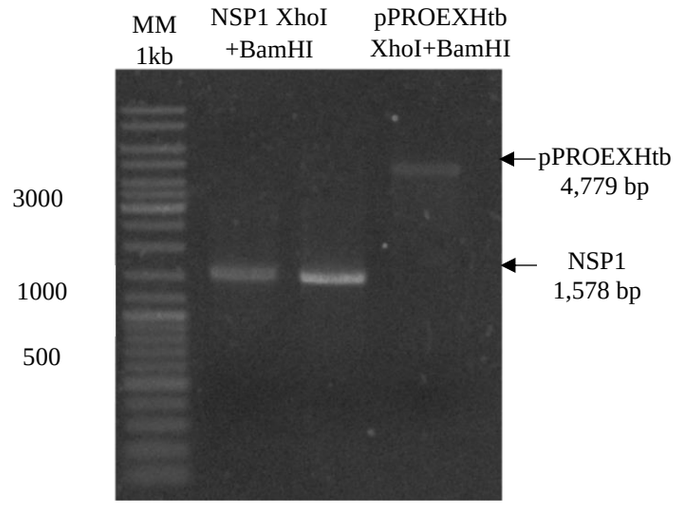
Besides, pJet blunt vector was selected since it can store large DNA sequences. Once the amplicon was ligated into the pJET transfer vector, its insertion was confirmed by digesting the construct with the *BglII* enzyme, which released the NSP1 gene, obtaining a fragment of the expected size (Figure 2.4).

Figure 2.4 pJET- NSP1 restriction electropherogram



As mentioned previously, to perform targeted cloning, both the expression vector pPROEX Htb and the NSP1 insert were digested with the restriction enzymes XhoI and BamHI, resulting in the release of fragments of expected sizes (Figure 2.5).

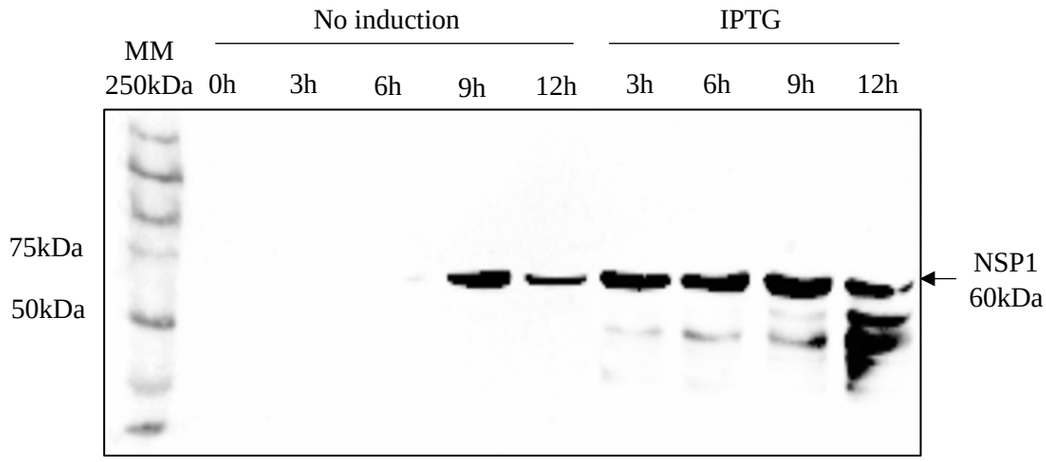
Figure 2.5 Electropherogram of the pPROEX Htb plasmid and amplicons digested with the restriction enzymes *Bam*HI and *Xho*I



2.3.2 Expression and identification of recombinant protein NSP1

Additionally, to confirm the functionality of the pPROEX Htb-NSP1plasmid, *E. coli* cells were transformed, and the culture was incubated with IPTG to induce the expression of the NSP1 protein. To evaluate recombinant NSP1 expression, western blot analysis was performed using an anti-His antibody to detect the protein. It was observed that a higher protein production occurred at nine hours post-induction (hpi) (Figure 2.6). Additionally, several smaller bands were observed at 6, 9, and 12 hpi that did not correspond to the product of interest (NSP1). The appearance of these bands may be due to NSP1 degradation since the stationary phase begins after several hours. During this stage, DNA and RNA synthesis are decreased, while protein synthesis is increased (Ramírez-Santos, 2005); thus, these bands may correspond to different sizes of NSP1. On the other hand, at 9 and 12 h, the bacterial cultures without IPTG showed small amounts of NSP1 due to a phenomenon known as “leaking,” which consists of incomplete repression of the expression of the protein. This is because many promoters are not very well regulated, showing little expression before the addition of an induction agent; *lac* promoters are highly likely to present this phenomenon (Miroux, 1996).

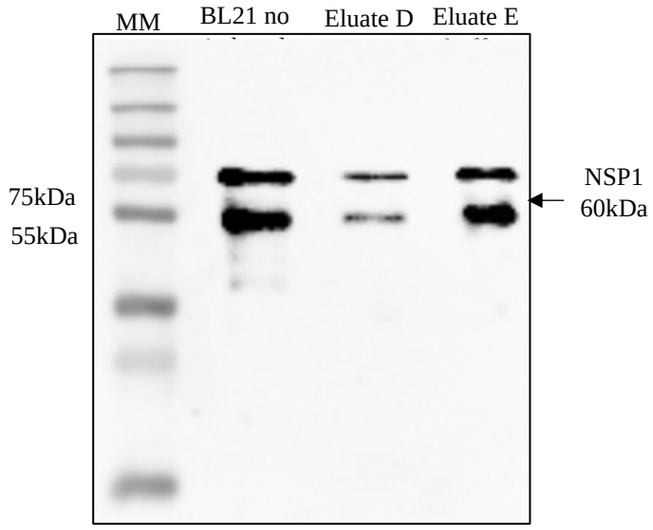
Figure 2.6 NSP1 expression kinetics in *E. coli.* Western blot analysis shows that the best time of protein production was at 9 h post-induction



2.3.3 Purification of recombinant NSP1

Furthermore, recombinant NSP1 was purified under denaturing conditions using histidine columns and then analyzed via western blotting. Two bands were observed in lanes 2 and 3 (Figure 2.7); the first band showed a molecular weight of 60 kDa, which was in accordance with the size predicted for NSP1, confirming the successful purification of the protein. However, a second smaller band was also observed, which may indicate a truncated product due to the presence of a premature stop codon. Interestingly, this doublet has not been reported in the literature for NSP1 in Chikungunya. Although it has been reported for other alphaviruses such as SINV, truncated proteins are still expressed even when there is a mutation in the reading frame (Akhrymuk, 2012).

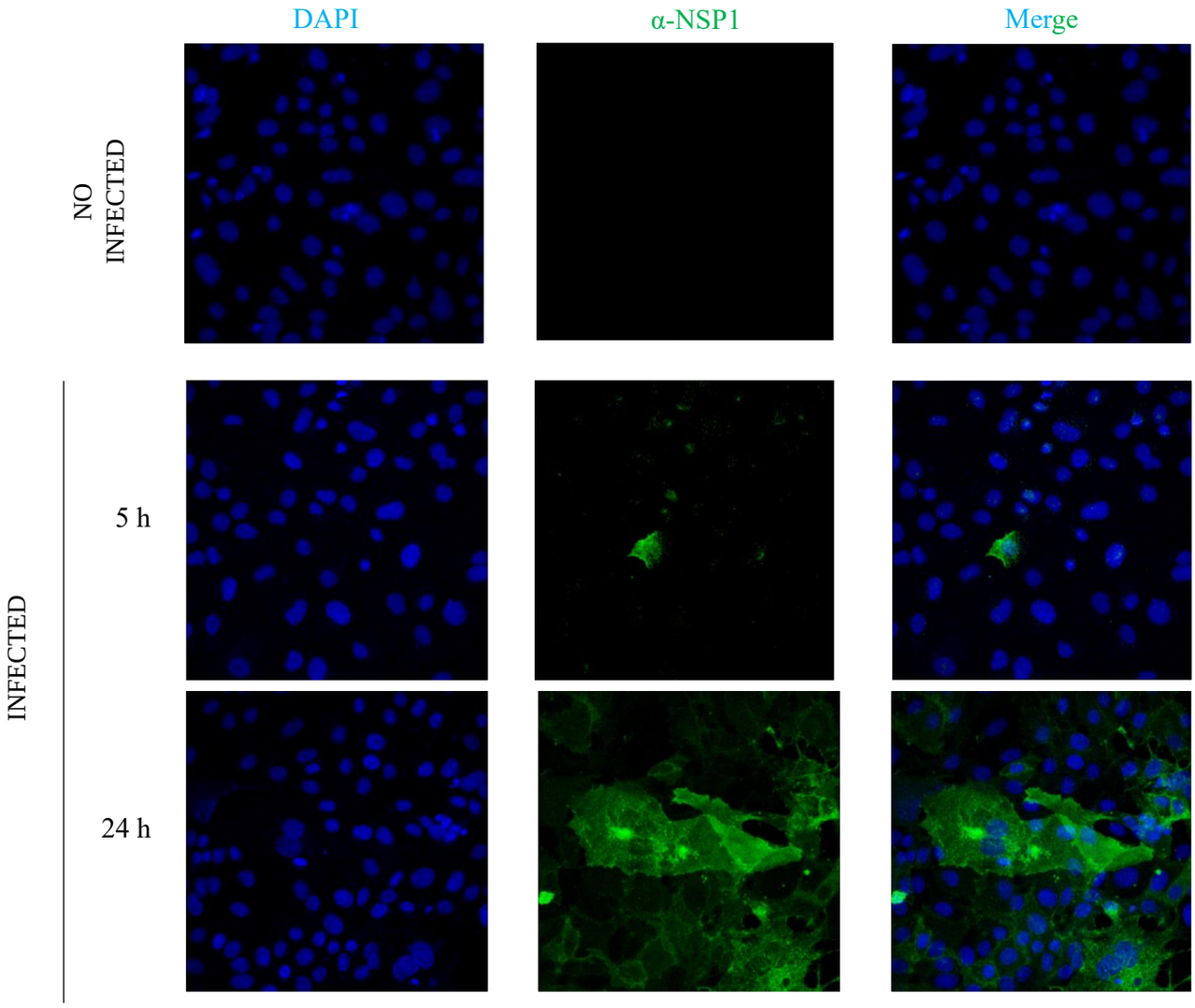
Figure 2.7 Purification of recombinant CHIK NSP1 using an Ni-NTA column. The eluted fractions were analyzed via western blot using a specific anti-His antibody



2.3.4 Evaluation of recombinant NSP1 to induce specific antibodies

Finally, recombinant NSP1 was used as an antigen to immunize rats, and the hyperimmune serum samples obtained were evaluated. We then addressed whether the antibodies raised in the immunized rats could recognize native CHIKV in infected Vero cells. Figure 2.8 shows Vero cells infected with CHIKV at an MOI of 5 and analyzed via IF at different times. The micrographs showed nuclei dyed with blue (DAPI) and green (FITC) staining was observed in the infected cells treated with the hyperimmune anti-NSP1 antibody. In contrast, the uninfected cells did not show the recognition of NSP1 by the antibody. However, during the first 5 h of infection, the positive signal was only faint, possibly because the production of NSP1 was not yet high. As previously reported, the transport of viral RNA is being carried out simultaneously with the synthesis of other nonstructural proteins (Kallio, 2015). However, at 24 h, the green signal is already stronger because late replication complexes, including genomic and subgenomic RNA, were already synthesized, and viral particles were released (Rana, 2014).

Figure 2.8 Specific recognition of NSP1 in Vero cells infected with CHIKV that were analyzed via immunofluorescence staining at 5 and 24 h post-infection. Serum samples from rats immunized with NSP1 were used as the primary antibody, and anti-mouse IgG-FITC was used as the secondary antibody. The Figure shows results from a representative experiment



One of the most relevant domains of the NSP1 protein is the membrane-binding domain, which allows its association with cell membranes. The presence of α-helical loops and palmitoylation sites in this domain allows the protein and replication complex to anchor to the plasma membrane (Abu Bakar, 2018). Hence, the presence of NSP1 was more pronounced in the cell periphery, as evidenced by the strong fluorescence observed in this area. Previously, Kumar et al. (2018) have shown that NSP1 is mainly found in the plasma membrane of cells infected with CHIKV (at an MOI of 2) at 2 hpi. In contrast, at 4 and 6 hpi, NSP1 can already be observed in the cell cytoplasm.

Furthermore, it has been previously reported that cells infected with CHIKV induce filopodia formation (Laakkonen, 1998) and cytoskeleton rearrangement (Karo-Astover, 2010). However, much work remains to be done to elucidate the changes induced by CHIK infection in host cells. Using the hyperimmune serum generated in this study, we can highlight and follow the drastic changes in cell morphology, mainly in the cytoplasmic membrane from where projections come out, as reported by Laakkonen and Karo-Astover (1998 and 2010, respectively).

Finally, it has also been reported that anti-NSP antibodies might be useful for investigating the stages of viral replication, describing the expression patterns of different viral proteins, or determining the location of NSPs in different cell compartments at certain times during infection (Kumar, 2015). Therefore, obtaining antibodies against the native form of CHIKV NSP1 could provide a valuable tool to investigate and understand the host-pathogen relationship in Chikungunya fever.

2.4 Acknowledgments

We would like to thank García-Cordero, Julio. PhD for the support in the elaboration of experiments shown in this project.

2.5 Founding

This work has been funded by CONACYT [grant number 2462-QLCB].

2.6 Conclusions

Recombinant NSP1 is an immunogenic protein, which allowed us to obtain polyclonal antibodies against rNSP1 that recognize the native form of NSP1. The antiserum is a powerful tool to study aspects of the viral cycle of CHIKV.

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