Original Article

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Development of a Novel Plasmid-based Eukaryotic Model to Investigate Crimean-Congo Hemorrhagic Fever Virus

Kırım-Kongo Kanamalı Ateşi Virüsünü Araştırmak için Plazmit Tabanlı Yeni Bir Ökaryotik Modelin Geliştirilmesi

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ABSTRACT

Objective: Crimean-Congo Hemorrhagic Fever (CCHF) is a severe tick-borne viral disease, caused by the Crimean-Congo Hemorrhagic Fever virus (CCHFV). The global expansion of CCHF and high mortality rates underline the critical need for research and development of effective treatments and vaccines. However, the high risk of transmission and requirement for high-containment facilities hinder investigations involving live virus. In this study, we aimed to address these challenges by employing a plasmid-based virus-like particle (VLP) system and a minigenome model to investigate the biology and immunology of CCHFV.

Methods: The plasmids encoding CCHFV structural genes of CCHFV were transfected into Huh-7 cells. Viral protein expression was confirmed using fluorescence imaging, immunological and molecular methods. A minigenome system was developed, eliminating the need for T7 polymerase, T7-expressing cellular lines, or viral ribonuclear protein complexes, allowing autonomous virus replication without a helper virus or transfections using plasmids in trans.

Results: Fluorescence microscopy showed successful production of NP-EGFP and GC-EGFP proteins with various subcellular localizations. Western blot analysis demonstrated the presence of pre-Gc, Gc, pre-Gn, Gn, and Np proteins in cell lysates and supernatants. ELISA analysis suggested that transfection of Np

ÖZ

Amaç: Kırım-Kongo Kanamalı Ateşi (KKKA), Kırım Kongo Kanamalı Ateşi Virüsü'nün (KKKAV) neden olduğu, kenelerle bulaşan ciddi bir viral hastalıktır. KKKA'nın küresel yayılımı ve yüksek ölüm oranları, etkili tedavilerin ve aşıların araştırılmasına ve geliştirilmesine yönelik kritik ihtiyacın önemini vurgulamaktadır. Bununla birlikte, yüksek bulaşma riski ve yüksek biyogüvenlikli tesislere duyulan gereksinim, canlı virüsle yapılan araştırmaları engellemektedir. Bu çalışmada, KKKAV'nin biyolojisini ve immünolojisini araştırmak için plazmid tabanlı virüs benzeri partikül (VLP) sistemi ve minigenom modeli kullanarak bu zorlukların üstesinden gelmeyi amaçladık.

Yöntemler: KKKAV yapısal genlerini kodlayan plazmitler, Huh-7 hücrelerine transfekte edildi. Viral protein ekspresyonu, floresan görüntüleme, immünolojik ve moleküler yöntemler kullanılarak doğrulandı. T7 polimeraz, T7 ifade eden hücresel hatlar veya viral ribonükleer protein komplekslerine olan ihtiyacı ortadan kaldıran ve bir yardımcı virüs olmadan otonom virüs replikasyonuna izin veren bir minigenom sistemi geliştirildi.

Bulgular: Floresan mikroskopisi, NP-EGFP ve GC-EGFP proteinlerinin çeşitli hücre altı lokalizasyonlarda başarılı bir şekilde üretildiğini gösterdi. Western blot analizi ile pre-Gc, Gc, pre-Gn, Gn ve Np proteinlerinin varlığı hücre lizatlarında ve süpernatanlarında gösterildi. ELISA analizi, yalnızca Np'nin, Np

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ABSTRACT

alone, in combination with Gc, or all three proteins might cause distinct VLP formations. Huh-7 cells successfully expressed reporter genes after transfection of minigenome RNA transcripts.

Conclusion: The study advances CCHFV research by using novel tools for virus biology and immunology. The findings may provide new avenues for research that promise better public health preparation against this neglected viral disease.

Keywords: Crimean-Congo Hemorrhagic Fever virus, virus-like particle, vaccines, bunyavirus, RNA virus infections, immunology

ÖZ

ile Gc'nin birlikte veya üç proteinin aynı anda transfeksiyonunun farklı VLP oluşumlarına neden olabileceğini göstermektedir. Ayrıca, minigenom RNA transkriptlerinin transfeksiyonu sonrasında Huh-7 hücreleri raportör genleri başarıyla açıklamıştır.

Sonuç: Bu çalışma, virüs biyolojisi ve immunolojisi için yeni araçların kullanılmasını sağlayarak KKKAV araştırmalarına katkı sağlayacaktır. Elde edilen bulgular kullanılarak, bu ihmal edilmiş viral hastalığa karşı halk sağlığı alanında daha hazırlıklı olma imkanları araştırılacaktır.

Anahtar Sözcükler: Kırım-Kongo Kanamalı Ateşi virüsü, virüs benzeri partikül, aşılar, bunyavirüs, RNA virüsü enfeksiyonları, immünoloji

Introduction

The etiological agents responsible for many of the human infections causing the millions of deaths are RNA viruses (1-5). Both the increased contact of humans with domestic animals and wild populations, brought on by globalization, as well as the high virulence and evolutionary plasticity of RNA viruses cause viruses to find new hosts. As seen with the emergence of AIDS, H1N1 or H5N1 flu, Nipah, Ebola and more recently coronavirus disease-2019, RNA viruses have triggered a series of pandemics originating from wildlife reservoirs, increasing the concerns on public health (6). In recent years, the World Health Organization (WHO) has published a list of diseases that should be primarily investigated. Due to its high potential for epidemic and public health emergency of international concern and the lack of effective therapeutics or vaccines for human or animal use, Crimean-Congo Hemorrhagic Fever (CCHF) is listed among these priority diseases for research and development in emergency contexts by WHO and as well as by National Institutes of Health (NIH) National Institute of Allergy and Infectious Diseases (NIAID) (7,8). The CCHF is a tick-borne viral hemorrhagic disease caused by Crimean-Congo Hemorrhagic Fever Virus (CCHFV) and causes sporadic outbreaks with mortality rates ranging from 5% to 80% (9,10). So far, virus isolation and/or disease have been reported in more than 30 countries over a wide geographical area covering Africa, Asia, and Eurasia (11). In 2019, WHO estimated that three billion people were at risk of contracting the disease because of the expanding transmission of the virus by Hyalomma ticks (12). Also, the CCHF became a growing public health problem in Türkiye, where the highest number of cases in the world were documented (11,041 cases between 2002 and 2018) (13).

The CCHFV belongs to the genus *Orthonairovirus* within the family *Nairovirideae* of the order *Bunyavirales* (14,15). The viral genome consists of three single-stranded negative-sense RNA molecules called L, M, and S segments that encode structural proteins such as RNA-dependent viral RNA polymerase, glycoproteins, and nucleoproteins (Np), respectively. Each contains a single open reading frame (ORF) and highly conserved non-coding terminal complementary sequences. The non-covalent interaction between both ends forms panhandle-like structure that provides a functional promoter region for viral polymerase binding (16-19). Encapsulation of the viral segments by Np by homo-oligomerization as pentamers and formation a ribonucleoprotein complex together with RNA-dependent RNA polymerase are crucial for the initiation of viral replication and transcription in the host cell (16). Two glycoproteins, Gn and Gc, are embedded in the lipid bilayer of the viral envelope and are responsible for viral attachment and internalization into the host cell (20). The maturation of glycoproteins begins with cotranslational cleavage of polyprotein into the glycoprotein precursors PreGn (140 kDa) and PreGc (85 kDa), likely by signal peptidase (21,22).

The CCHFV has been spreading to new regions and causing more frequent outbreaks in recent years. It is considered as a neglected disease, and efforts are needed to improve our understanding of CCHF pathogenesis and the diagnosis, treatment, and prevention of the disease. The high risk of transmission and the necessities for BSL-4 facilities hamper the studies with live virus. So, any information on virus biology, especially that provided by individual proteins, will be a valuable contribution to the knowledge on CCHFV.

Understanding the life cycle of a virus is critical for the development of vaccines and antivirals, and the establishment of robust methods plays a critical role in investigating the underlying molecular mechanisms of the disease. Reverse genetic systems are effective tools that enable mutation analyses, which help determine the molecular basis of the viral life cycle in all its aspects (23). While viral genome replication and transcription are modeled using minigenome systems, the morphogenesis, budding, and infection of target cells are simulated using virus-like particles (VLPs), which shed light on unknown mechanisms of the virus life cycle and the pathophysiology of diseases. The absence of genomic material facilitates research on such viruses in BSL-2 laboratories, which are easily accessible to many researchers worldwide. Therefore, here it was aimed to produce viral proteins in mammalian expression systems to establish a plasmid based VLP system which could contribute to existing knowledge about CCHFV biology and immunology. With this study, it was also aimed to develop a minigenome system that could be utilized in the other studies aiming to generate VLP models which could

simulate one cycle of virus replication. Unlike studies in the literature, it was aimed to develop a unique minigenome system that eliminated the need for both T7-expressing cell lines and a viral ribonuclear protein complex (RNP) generally aided by either a helper virus or by plasmid transfections in trans. Thus, the expression studies conducted here may further facilitate studies of the virus life cycle, the discovery of new therapeutics, and the development of diagnostic tools.

Methods

Cell Culture and Virus

Human hepatocellular carcinoma cells (Huh-7) were grown at 37 °C with 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 100 units/mL penicillin, and 100 µg/mL streptomycin.

Antibodies and Sera

Mouse monoclonal antibodies targeting CCHFV strain Ibar10200 anti-PreGn/GP38 (clones 8F10), anti-PreGc (clone 11E7), and anti-NP (clone 9D5) and were obtained from the Joel M. Dalrymple-Clarence J. Peters USAMRIID Antibody Collection through BEI Resources, NIAID, NIH. The CCHFV-immunized rabbit and mouse sera were kindly given by Prof. Aykut Özdarendeli (Erciyes University Vectors and Vector Borne Diseases Implementation and Research Center, and Department of Medical Microbiology, Faculty of Medicine, Erciyes University, Kayseri, Türkiye).

Construction of Plasmids

The nucleotide sequences of CCHFV Kelkit06 S (1673 nt) and M (5364 nt) segments retrieved from GenBank (accession numbers: GQ337053 and GQ337054, respectively) were synthesized with flanking *Sap*I recognition sites at both ends and cloned separately by *Kpn*I and *Bam*HI enzymes into pUC19 vector (Synbio Technology USA Inc., Suzhou, China). The constructed plasmids (named as pUC_S and pUC_M) were propagated in One Shot™ TOP10 Chemically Competent *E. coli* (ThermoFisher Scientific, USA) cells. The ORFs of CCHFV Kelkit06 Np, PreGn and PreGc were cloned into pcDNA 3EGFP (13031, Addgene) and pcDNA3-neo-cterminal-3HA (102643, Addgene). The constructed plasmids were transformed into XL10-Gold™ ultracompetent *E. coli* cells (Agilent, CA, USA). Three positive transformant colonies for each construct were selected and analyzed by restriction digestion analysis. The minigenome plasmid 7miniC was commercially syntesized (Synbio Technology USA Inc., Suzhou, China) and propagated in One Shot™ TOP10 Chemically Competent E. coli cells. The eGFP gene was replaced with mCardinal to generate 7miniE plasmid by using directional cloning utilizing *Kpn*I and *Xho*I enzymes.

Transfections

One day before transfection $5x10^5$ cells/well were seeded in 6-well plates and grown in DMEM (Gibco™, ThermoFisher Scientific, USA) supplemented with 10% fetal calf serum, (Gibco™, ThermoFisher Scientific, USA) and Pen-Strep (Penicillin-Streptomycin, Gibco™, ThermoFisher Scientific, USA) at 37 °C and 5% CO_2 in a humidified chamber until they reached 50-60% confluency on the day of the experiment. The next day, cell culture media was removed, and the cells were incubated with DMEM including 10% FBS with Pen-Strep. Meanwhile, polyethyleneimine (PEI) (Polysciences, Germany) complexes were prepared. Briefly, 6 µl of 1 mg/mL PEI was diluted in 100 µl DMEM (-), then mixed with 2 µg plasmids diluted in 65 µl DMEM (-) (1:2 ratio, 15 ng/µl DNA: 30 ng/µl PEI). Then PEI complexes were added, and the cells were incubated for 72 hours at 37 °C and 5% CO_2 in a humidified chamber. Three days post-transfection, cell culture supernatants and pellets were collected and stored at -20 °C for further analysis.

Cell Lysis

Transfected cells were washed two times with cold PBS and were collected by scraping followed by centrifugation at 1000 g for 3 min. The cell pellets were lysed in lysis buffer (20 mM Tris pH7.5, 1% Triton X-100, 0.05% SDS, 0.5% Sodium Deoxycholate, 150 mM NaCl, 1mM Protease inhibitor cocktail).

RT-PCR

Total RNA was isolated from cells post-transfection using TRI-Reagent (BioShop, Canada) and samples were treated with DNAse I for 15 min at 37 °C and then purified from DNAse I. RT-PCR was performed by the SensiFAST™ cDNA Synthesis Kit (Bioline, UK) using the Oligo d(T) primer to generate cDNA. Then qPCR was performed via of BlasTaq™ 2X qPCR MasterMix using different primer sets that could amplify approximately the last 130 bp of ORF sequences for each of the viral genes. The qS forward (5' CATACAGGACATGGACATTGTG 3') and qS reverse (5'TTAGATGATGTTGGCACTGGTG 3') for Np; qM forward (5'CAGGCTACAGAAGGATTATTGAAAGAC3') and qM reverse (5'TTAGCCAATGTGTGTTTTTGTGGAG 3') for Gc; Primer 8 forward (5' CTTGGTACCGCCACCATGGTCTGCAAACGC3') and Primer 3 reverse (5'GTGGATCCTTATGCAGAGGTGCTAAC 3') were used for Gn.

Western Blot

The proteins to be analyzed were separated on either by SDS-PAGE or Native-PAGE, described below.

Polyacrylamide Gel Electrophoresis

SDS-PAGE: The protein samples were diluted 1:1 in Laemmli buffer [4% (w/v) SDS, 10% (v/v) β-mercaptoethanol, 20% (v/v) glycerol, 0,004% (w/v) bromophenol blue, 0,125 M Tris-HCI] and heated to 95 °C for 10 min unless otherwise stated. For some assays, Np and Gn samples were denatured in reducing loading buffer (5X Blue Loading Buffer, 200 mM Tris HCl pH6.8, 10% SDS, 500 mM β-mercaptoethanol and 50% glycerol) at 95 °C for 5 min. Proteins were run on an 8-12% polyacrylamide gel, first at 50 V for 20 minutes, then at 200 V for 40 minutes. After electrophoresis, the gel was stained with Coomassie brilliant blue G-250 (Sigma-Aldrich, Taufkirchen, Germany).

Native-PAGE: Samples were diluted in non-reducing loading buffer (5X Blue Loading Buffer, 200 mM Tris HCl pH6.8, 20% SDS, and 50% glycerol), incubated at 50 °C for 10 min and electrophoresed on 4-9% polyacrylamide gels.

Following electrophoresis, proteins were transferred to a polyvinylidene fluoride membrane (GVS North America, Sanford, USA) immediately after methanol induction of membrane, in transfer buffer [24 mM Tris, 192 mM glycine and 20% (v/v) methanol] either at 25 V for 7 minutes on semi-dry blotter (Trans-Blot Turbo, Bio-Rad, California, USA) or wet at 100 V for 90 minutes in Mini Trans-Blot cell (Bio-Rad, California, USA). The membrane was blocked with TBST [10 mM Tris pH 7.4, 0.9% (w/v) NaCl, and 0.05% (v/v) Tween-20] containing 5% (w/v) skim milk (Sigma-Aldrich, Taufkirchen, Germany) for two hours at room temperature and incubated with primary antibody overnight at 4 °C, followed by secondary antibody incubation for one hour at room temperature. Between each step, the membrane was washed five times of 10 min using TBST and processed for chemiluminescence detection (WesternBright Sirius Chemiluminescent Detection Kit, Advansta, California, USA), according to the manufacturer's instructions. Membranes were visualized by using Fusion FX Solo imaging system (Vilber, Collégien, France).

ELISA

The 96 well microplates (Immulon 2 HB, Invitrogen, Waltham, USA) were coated overnight at 4 °C with supernatant or cell lysate samples diluted in carbonate-bicarbonate buffer (pH 9.6). Then, the plates were washed four times with PBST [PBS + 0.05% (v/v) Tween 20] and blocked with PBST containing 5% (w/v) skim milk for two hours at room temperature. Plates were then incubated at 37 °C for 1 hour each, first with the primary antibody and then with a corresponding HRP-conjugated secondary antibody, depending on the analytes. Between each step, the wells were washed four times with PBST. Then, TMB solution (Abcam, Cambridge, UK) were added to the wells and the plates were incubated for 20-25 minutes at room temperature in the dark. The reaction was stopped by adding 2N H_2SO_4 to the wells and absorbance of samples measured in iMark microplate reader (Bio-Rad, California, USA) at 450 nm. All washing steps performed using Wellwash Versa Microplate Washer (Thermo Fisher Scientific, Waltham, USA). Data were calculated by the mean of absorbance measurements obtained from duplicated samples. The cut-off value was calculated by the given formula: the mean absorbance of each negative samples + 2 SD.

***In Vitro* Transcription**

The minigenome cassettes were excised by *Eco*RI enzyme and used as template for in vitro transcription. Then it was processed by using HiScribe® T7 High Yield RNA Synthesis Kit (New England Biolabs, MA, USA), according to the manufacturer's instructions.

Statistical Analysis

For the analysis of results obtained from RT-PCR, Ct values measured by Rotor-Gene® Q (Qiagene, USA) for each gene and were normalized to the *GAPDH* gene used as internal control. The relative expression level was analyzed by 2^{-DDCt} method using the following formula:

$$DDCt:[(Ct_{targetgene}-Ct_{housekeeping})-(Ct_{normal}-C_{thousekeeping})]$$

ELISA data were calculated by the mean of absorbance measurements obtained from duplicated samples. The cut-off value was calculated by the given formula: the mean absorbance of each negative samples + 2 SD.

Results

For the establishment of a plasmid based CCHFV VLP model in eukaryotic cells, pCDNA3.1 plasmids encoding Np, PreGn and PreGc regions were utilized to express viral proteins in Huh-7 cells. Fluorescence microscopy images at 48 hours post transfection showed that both NP-EGFP and GC-EGFP proteins were successfully produced and had various subcellular localizations (Figure 1). While Gc was uniformly distributed across the cell surface, Np was primarily found in the perinuclear region. These different expression profiles

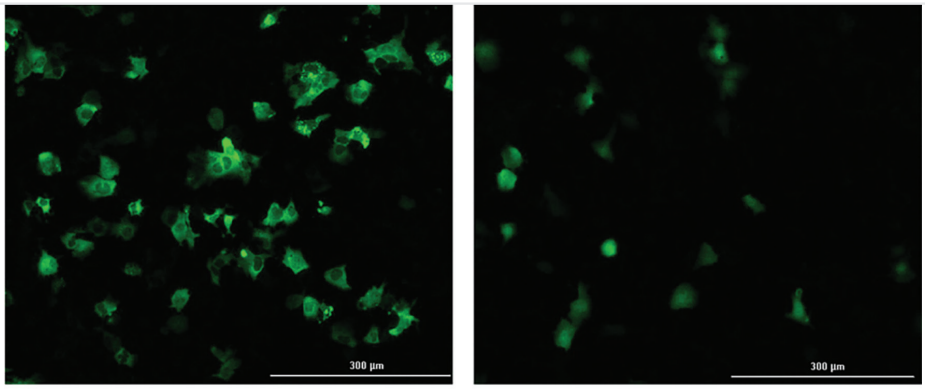

Figure 1. The visualization of differential cellular localization of NP-EGFP and GC-EGFP proteins in Huh-7 cells under fluorescent microscopy

*Scale bar 300 µm

might imply that viral proteins were expressed recombinantly and functioned properly.

As the presence of viral proteins was indirectly demonstrated by fluorescence microscopy, additional expression analyzes were performed to assess production. Proteins were first analyzed by ELISA method, in which the supernatant samples from transfected Huh-7 cells were analyzed using CCHFV-immunized mouse and rabbit sera, human convalescence sera, and CCHFV-specific mouse mAb antibody cocktail including anti-NP (9D5), anti-GN (8F10), and anti-GC (11E7) primary antibodies. It was observed that positive samples had almost similar pattern in all tests (Figure 2). All antibodies detected the viral proteins in the samples of "3in1", in which all viral plasmids were transfected simultaneously. The reproducibility of this results suggested that CCHFV VLP might be formed. Remarkably, each test consistently yielded positive results for Np + Gc cell culture samples, indicating the potential ability of Np and Gc to form a VLP structure. The Gn was also tested positive in all assays except specific antibody ELISA, suggesting that Gn might be able to be packed alone in the Golgi, due to its Golgi retention signal on their sequence. Additionally, Np was detected in all assays tested here, which might provide additional evidence to the previous study reporting that Np formed sphere-like structures in cytoplasmic vesicles after its expression using baculovirus expression system (24). This analyzes showed that viral proteins were successfully expressed and some of their combinations might contribute to form VLPs in transfected cells.

To confirm viral protein production in transfected cells, further analyzes were conducted by western blot assays (Figure 3). When the membranes were incubated with CCHFV-immunized mice sera after SDS-PAGE, pre-Gc (85 kDa) and Gc (75 kDa) were detected in the lysates while bands around 130 kDa and 245 kDa for Gn were appeared in cell culture supernatant samples (Figure 3A). Meanwhile, Np (53 kDa) proteins were detected both in lysate and supernatant samples, when they transferred to the membrane after Native-PAGE (Figure 3B). Finally, all three proteins Np, Gn, and Gc were recognized by the antibodies found in the CCHFV-immunized rabbit sera (Figure 3C). Also, post-transcriptional analysis of viral proteins revealed at least 2x104 fold increase in viral gene expression compared to the GAPDH (Figure 3D). Accordingly, these results indicated that viral proteins were successfully expressed in Huh-7 cells

To study viral replication and transcription, as well as entry, assembly, and budding of CCHFV, a transcriptionally competent VLP model is required. To achieve this goal, a minigenome plasmid is needed to be packaged into the VLP, such as the CCHFV segment. With a unique strategy, an ambisense minigenome plasmid was so designed that might transcribe vRNA in VLP forming donor cells without using any viral polymerases. The minigenome plasmid named as the 7miniC has ambisense vRNA coding cassette, a reporter gene in positive polarity was located between the antisense 3'untranslated region (UTR) and 5'UTR sequences of the CCHFV L segments and downstream of the T7 promoter region was encoded and two additional UTR sequences (NEO UTR2 and NEO UTR 3) in sense orientation were inserted upstream of the reporter gene to increase its expression. To generate synthetic RNAs with a precise 3' end, the hepatitis delta virus ribozyme sequence was added to the final nucleotide of the viral genome (Figure 4A). To demonstrate proof of concept, in vitro transcribed minigenome RNA transcripts, carrying either mCardinal (7miniC) or eGFP (7miniE) as reporter gene were transfected into Huh-7 cells using Lipofectamine or PEI. The expression of reporter gene was visualized by laser scanning confocal microscopy (Figure 4B). In this study, a unique ambisense minigenome system was developed for CCHFV. Unlike existing systems, the fact that this new minigenome system does not require additional helper virus or viral RNP complex has brought an innovative approach for CCHFV reverse studies.

The CCHFV is a highly pathogenic virus with increasing prevalence. In this sense, the development of a safe models that simulate the virus infection particularly such as replication competent VLPs has a vital role in virology. In this study, viral proteins of CCHFV Kelkit 06 strain were expressed in

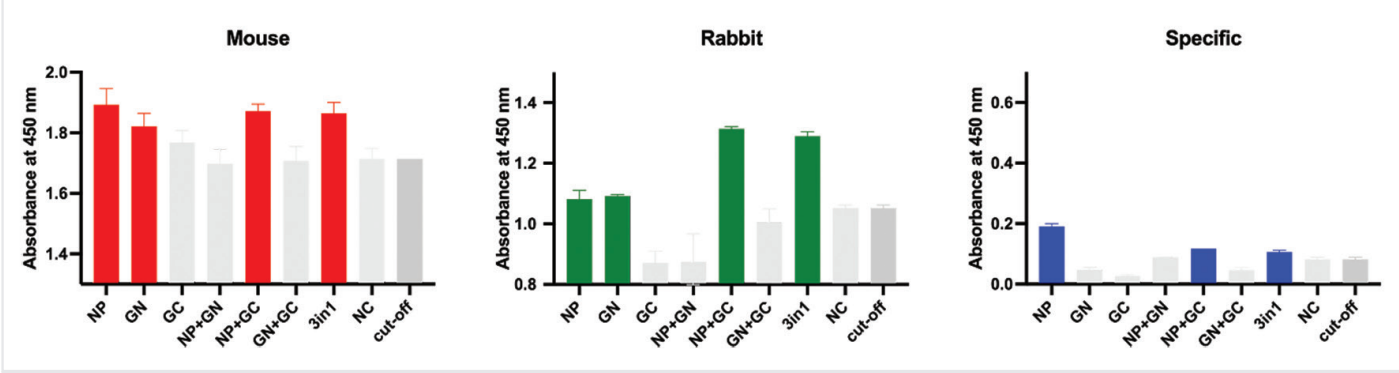

Figure 2. Analysis of supernatant samples of the Huh-7 cell transfected with different combination of viral plasmids, by in house ELISA tests

The supernatant samples were probed with CCHFV-immunized mouse (red), and rabbit sera (green), and with specific monoclonal antibody cocktail. The absorbance of each sample was measured at 450 nm using iMark microplate reader (Bio-Rad, California, USA).

CCHFV: Crimean-Congo Hemorrhagic Fever Virus

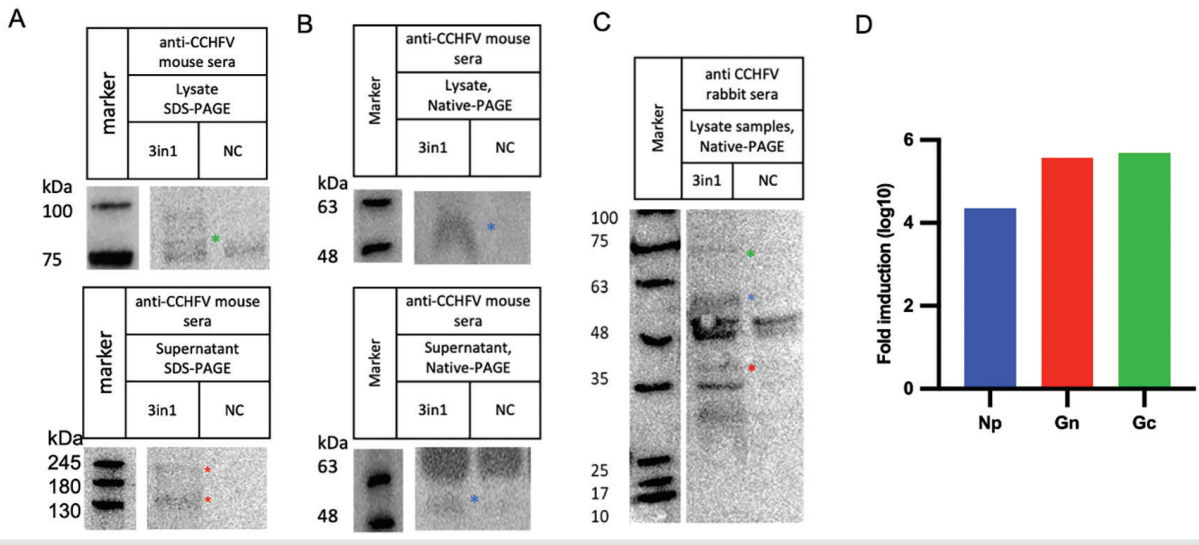

Figure 3. Analysis of viral protein expression

Western blot analysis of viral proteins in Huh-7 cells, transfected with all plasmids together. Lysate and supernatant samples analyzed using CCHFV-immunized mouse sera were either dissolved on SDS-PAGE (A) or on Native-PAGE (B), while lysate proteins dissolved on Native-PAGE were analyzed using CCHFV-immunized rabbit sera (C). (D) Post-transcriptional analysis of viral mRNAs by RT-PCR. The relative gene expression levels to GAPDH, as an internal control were measured using the $2^{-\Delta\Delta CT}$ method

CCHFV: Crimean-Congo Hemorrhagic Fever Virus

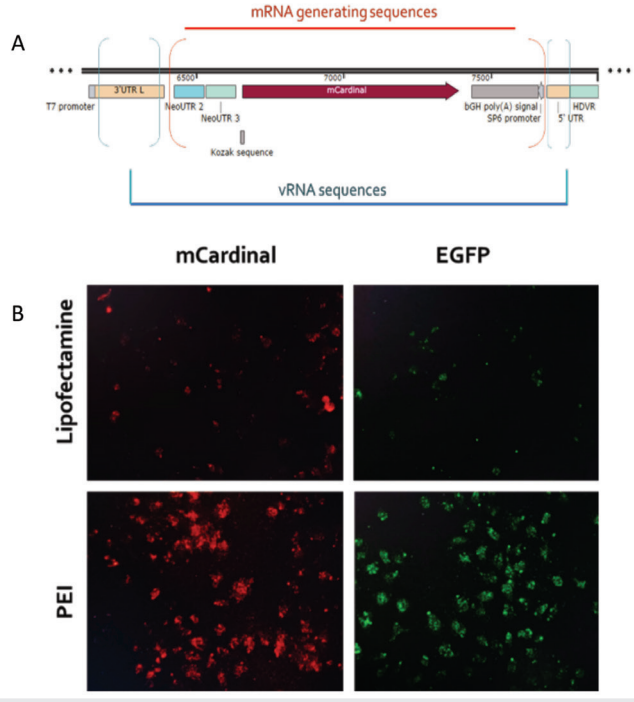

Figure 4. The design and the proof of concept of a novel CCHFV minigenome model

A) Partial vector map showing the ambisense vRNA-encoding cassette in 7miniC plasmid. The mRNA generating sequence, in which ORF of reporter gene together with the regulatory sequences, recognized by human protein translation system was inserted between negative sense viral UTR sequences, providing viral genome packaging signal. B) Confocal laser scanning microscopy images of Huh-7 cells transfected with 7miniC (red) and 7miniE (green) *in vitro* transcripts using PEI or lipofectamine at 6 hours post-transfection

CCHFV: Crimean-Congo Hemorrhagic Fever Virus, PEI: Polyethyleneimine

mammalian cells, which had the potential for development of such a VLP model. In addition, the inclusion of the newly developed minigenome system in future VLP studies will pave the way for studies on both the life cycle of CCHF and the development of antiviral treatment methods.

Discussion

Our study demonstrated that viral proteins were produced in Huh-7 cells and the expression was confirmed by immunological and molecular methods. The model developed in this study promised to be utilized in future biology, immunology, and vaccinology efforts of CCHFV. Fluorescent microscopy images revealed that eGFP displayed various intracellular signals depending on the viral protein with which it was fused. When eGFP was expressed as a fusion with Np, it fluoresced in the perinuclear region, while a diffuse fluorescent light throughout the cytoplasm was noticed when it was fused with Gc. In the literature, CCHFV Np has been reported to localize in the perinuclear region of infected cells (25-27) and Gc requires Gn for its transition to the Golgi (28). In accordance with the information in the literature, fluorescence microscopy assay provided the first data suggesting efficient production of Np and Gc proteins. Immunological analysis of viral proteins by western blot demonstrated that all viral proteins were expressed in Huh-7 cells. Furthermore, cell culture samples were examined using ELISA, and samples in which all Np, or a combination of Np and Gc, or all three proteins simultaneously were transfected were recognized by all antibodies tested in this study. According to current knowledge, the Np of Dengue virus-2 and of Hepatitis C virus can self-assemble into VLPs (16,20). Furthermore, Zhou et al. (24) reported that CCHFV Np form sphere-like structures that vary in morphology and size, ranging from 40 nm to 160 nm in diameter, within cytoplasmic vesicles of Np transfected insect cells. Our findings suggested that the model developed in this study opened the possibility to investigate the VLP formation the minimal protein composition needed for CCHFV VLP.

One aim of the study was to generate a minigenome system that could be integrated into the CCHFV VLP system. Several minigenome systems have been developed for bunyaviruses (23). The viral polymerase and Np are minimally required components for a proper genome replication and transcription in bunyaviruses (29). However, due to the difficulty of cloning 12 kb CCHFV viral polymerase gene, a unique minigenome system was designed that could simultaneously generate a single RNA that possessed the features of both vRNA of CCHFV and mRNA of a reporter gene, without the aid of any viral RNP complexes. Unlike existing systems, this system requires neither T7 expressing cells, Pol I, nor viral polymerase. This minigenome cassette has ambisense character since an mRNA cassette in sense orientation has been flanked by antisense viral UTR sequences on the same strand. Similarly to the previously reported Lassa virus minigenome system (30), the signal-to-noise ratio was significantly improved by transfecting *in vitro* synthesized minigenome RNA rather than plasmid DNA utilizing either T7 or Pol I polymerases (31). On the other hand, VLP production independent of a permanently T7 polymerase expressing cells (like BSR-T7/5) might provide versatility in CCHFV studies with various cells, as well. For this reason, the minigenome RNA used in this study was synthesized in vitro using T7 polymerase. Confocal microscopy images showing the expression of reporter genes in Huh-7 cells transfected with in vitro transcribed minigenome RNA proved that our model allowed the generation of reporter proteins without any viral RNP complexes, which were generally required for most minigenome systems. As a result, this model might enable one to scrutinize steps of the viral life cycle, such as virion assembly, genome packaging, and cell entry mechanisms, once the viral structural proteins provided in trans.

The invaluable contribution of reverse genetic studies involving VLP systems, particularly with the integration of minigenomes to the biology of CCHFV are demonstrated during the last decades (32-37). To combat CCHFV effectively, it is important to conduct studies on characterization of biological, immunological, and pathogenic features of the virus in a safe manner. The purpose of the current study was to develop such model. Here, experiments utilizing mamalian expression vectors were conducted to generate a VLP systems for CCHFV. Viral protein expressions were demonstrated in tests involving immunological and post-transcriptional expression analysis methods. Additionally, a viral RNP complex-independent ambisense minigenome system was developed for future studies, aiming to generate cell-entry competent and transcriptionally active VLPs for CCHFV Kelkit 06 strain. It is believed that the data generated in this study will pave the way for future comprehensive studies investigating each stage in the life cycle of the virus and as well as developing diagnostic, protective, and therapeutic agents against CCHFV.

Study Limitations

The results obtained from the preliminary studies required for CCHFV VLP production in this study showed that viral proteins of CCHFV Kelkit 06 strain could be produced in Huh-7 cells using pcDNA 3.1 plasmid. However, the model was not sufficiently driving the production of VLPs by which some of the virological questions could be addressed. It appears that optimization of the model is required. It appears that for generation and demonstration of sufficient quantities and commercially meaningful VLPs from plasmid based expression systems, further optimizations are needed. It should be underlined that to address the questions about the biology and immunology of these viruses through VLP and minigenome approaches, optimizations with different expression models, sequences, and transfection systems should be undertaken. Furthermore, stable expressions in susceptible cell lines could also be useful.

Conclusion

The experiments conducted in this study showed expression and detection of immunologically significant viral antigenic proteins from CCHFV. Although ELISA results imply that various viral protein combinations produce different VLP compositions, further confirmatory studies are needed to support the results

obtained in this study. As another objective of this study was to form a viral RNP complex-independent ambisense minigenome system to generate transcriptionally and entry-competent VLP for CCHFV Kelkit 06 strain. For this aim, the expression studies in the study contributed valuable information in the development of VLP systems. This information might pave the way for future studies to be conducted to comprehensively investigate each stage in the life cycle of the virus and as well as to develop vaccine and treatment agents against CCHFV.

Ethics

Ethics Committee Approval: Ethic consent is not required.

Informed Consent: Informed consent is not required.

Authorship Contributions

Concept: N.S.G.Ç., M.Z.D., Design: N.S.G.Ç., M.K.Y., M.Z.D., Data Collection or Processing: N.S.G.Ç., Ö.B., S.K., Analysis or Interpretation: N.S.G.Ç., Ö.B., M.K.Y., Literature Search: N.S.G.Ç., S.K., Writing: N.S.G.Ç., M.Z.D.

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