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Original Scientific Paper

Optimization of HCV cell culture system for evaluation of antiviral drugs against hcv genotypes 2a and 4a

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SUMMARY – Evaluation of hepatitis C virus (HCV) infection in cell culture is important for development of new antiviral agents and for understanding the viral life cycle, as well as the correlation among the genotypes spreading worldwide. Development of HCV cell culture systems offers great advantage by enabling growth of the virus in cell culture, allowing for discovery of potential therapeutics and vaccines. The aim of this study was to introduce the steps of HCV infection in the cell culture system as a determinant for the competence of more potent antiviral compounds. Two plasmids, JFH1 (genotype 2a) full length and 4a (ED43/C-NS2/NS5A) were subjected to *in vitro* transcription for HCV RNA synthesis followed by transfection of Huh7.5 with the resulting RNA from each genotype processed to generate the virus. Later, Huh7.5 cells infected with the replicated virus resulted in the generated HCV infected cells. Immunostaining of the infected cells showed fluorescence of green color, indicating positive infection of both genotypes 2a and 4a. In addition, real-time polymerase chain reaction was performed to measure viral load from extracted cells previously infected with the RNAs of genotypes 2 and 4, which resulted in 32,500 and 107,375 copies/mL, respectively. In conclusion, introduction of HCV cell culture system is a convenient method to evaluate active antiviral drugs against HCV genotypes 2a and 4a using both qualitative and quantitative techniques in distinguishing the effectiveness of antiviral agents.

Key words: *Hepatitis C virus; Infection; Huh7.5; JFH1; Genotype 4*

Introduction

Hepatitis C virus (HCV) is a significant global health problem that can cause severe complications and long-term health issues. Development of direct-acting antivirals (DAAs) was a revolutionary step toward progression of HCV treatment options. There are only a few limitations and restrictions for these drugs such as hepatic decompensation, progressed stages of chronic kidney disease, and aberrant renal function. Also, there are no limitations to treating HCV infection in liver transplant recipients. Therefore, antiviral treatment in a safe and efficient manner is required after transplantation of the kidney, lung, and heart[1]. However, the efficiency of antiviral treatment can greatly vary in some less susceptible genotypes. In order to overcome

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this challenge, it is essential to expand the knowledge regarding the virus and its various genotypes to develop effective treatments and preventive measures.

One of the crucial methods for studying HCV are cell culture models. Cell culturing has been recognized as one of the most valuable methods in basic and translational medical research. Mostly, cell culture models are used for studying the pathophysiology of various diseases, as well as drug toxicity. The lack of interference of environmental influence, merely no ethical concerns, and the possibility of gene and/or molecular manipulation is one of the major advantages of these systems. Cell culture model of HCV infection can be established for multiple purposes. In virology, the process of incubating and cultivating viruses within cell cultures under controlled and favorable conditions has proved to be a highly effective method of detecting and identifying various viral pathogens while allowing a study design suitable for researching the virus life cycle, replication, and interaction with host cells. This model has also become a valuable tool of studying the antiviral effects of various potential treatments, including plant extracts, chemical compounds, drugs and vaccines[2]. Recent studies investigated HCV infection and its correlation to host innate immunity, as well as how successful cell culture system studies could contribute to new insights in anti-viral treatment, vaccine development, and overall improvement of health in patients[3-5].

The HCV genome consists of 9,600 nucleotides with about 9,000 single open reading frames (ORF) flanked at the terminal with critical points for viral replication and translation, consisting of 50 and 30 untranslated regions (UTR). HCV ORF translation produces a polyprotein product of just over 3,000 amino acids, cleaved by both host and viral proteases, resulting in three structural proteins that form the virus particle. These three structural proteins include the core protein and envelope glycoproteins E1 and E2. Additionally, seven nonstructural proteins (p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B) are responsible for viral processing and replication, as well as particle assembly. HCV belongs to the *Flaviviridae* family of viruses, which have a positive-sense single-stranded RNA genome and similar organization. The viral genome functions as a template for replication in the host cell, through the association of messenger RNA with modified cell membranes by negative-strand full-length intermediates[4,5].

Current antiviral therapies using DAAs have a lower rate of cure in patients infected with genotype 3 compared to other genotypes, and these patients are at a higher risk of developing hepatic steatosis. This has led to an increased focus on the discovery of new antiviral agents[6]. Efficient *in vitro* systems for HCV replication research have been established, including the model of JFH1 strain belonging to genotype 2a that has exceptional replication ability in Huh7 cells. Intergenotypic recombinants of JFH1 were established, comprising the structural genes Core, E1, and E2, as well as p7, and complete or parts of NS2 of the 4a prototype ED43 strain. Following transfection of Huh7.5 cells with RNA transcripts, production of the infectious virus was achieved[7]. In 2009, full-length HCV RNA transfection into Huh7 cells for the production of an infectious virus was reported, following isolation of a JFH1 genotype 2a strain from a patient with fulminant hepatitis C[8]. It was able to replicate in Huh7 cells and derivatives without the need of adaptive mutations[9]. Replication of other HCV strains required some modification particularly comprising introduction of cell culture-adaptive mutations. The sub-genomic replicon assay was screened for adaptive mutations followed by the introduction into the full-length genomes. Transfection of the cells with the resulting viral RNA was repeated in the passage until generation of the virus particles[10].

The advances and developments in HCV cell culture systems started with a replicon system published in 1999. The reading frame was completed through the amplification of two overlapping fragments using real-time polymerase chain reaction (RT-PCR). The replicon includes the HCV 5" non-coding region linked to the first amino acids of the core protein, the neomycin phosphotransferase coding region that provided geneticin antibiotic resistance resulting from the internal ribosome entry site (IRES) of HCV[11]. As for genotype 1b, 1a and 2a isolates that developed replicons, one genotype 2a replicon was able to replicate about 20-fold more effectively than genotype 1a despite the lack of any required cell-culture adaptive mutations. Genetically engineered viruses are used in cell culture systems to obtain growth in culture by means of the replicon, but the advances in natural isolate usage have improved HCV culture research[12].

As for recent advances in HCV cell culture systems, a genotype 1b isolated variant from the serum of a very high titer patient after transplantation of liver (German Liver Transplant 1, GLT1) was able to show exceptionally efficient replication of the genome in hepatoma cultured cells. The isolate was adapted for the production of infectious virus, thus leading to the generation of a novel cell culture model for HCV genotype 1b with a full-replication cycle. The adapted GLT1 remains infectious *in vivo* even with multiple mutations[13].

The emerging of an alternative system, trans-complemented JFH1 particles (HCVTCP) offers a method for studying the entire life cycle of HCV, that could potentially overcome the issue of isolate specificity. To generate HCVTCP, two components are necessary, i.e., a JFH1 subgenomic replicon that enables virus replication and an expression system for structural proteins of HCV, as well as p7, and NS2. This system provides genotype-specific information regarding the ways in which the virus enters cells, replicates, and assembles, and allows for deeper understanding of the entire HCV life cycle. By using specific adaptive mutations and custom-designed packaging systems, the system could overcome limitations in producing viral particles due to genetic incompatibility between the replication module and the packaging cassette, which can vary among different HCV genotypes. As a result, the production efficiency of HCV particles in HCVTCP system is less restricted by genotype-specific differences[14]. Many HCV-related hepaciviruses have been detected due to advances in sequencing technology[15].

In this study, both JFH-1 full length and ED43/C-NS2/NS5A plasmids for genotype 2 and 4, respectively, were selected for optimizing the parameters required for effective generation of infectious virus. As successful trails for JFH-1 infectious particles production since 2006 opened the widespread optimization protocols for HCV cell culture systems, infections with HCV-genotype 4 in the Middle East/Africa were recorded to be associated with the increased rate of treatment failures shown with DAA. The goal of this research was to illustrate the steps for an efficient HCV cell culture system for two HCV genotypes (2a and 4a) that could be utilized to test anti-HCV medicines and HCV vaccine candidates.

Materials and Methods

Cell culture

Huh7.5 cell line, a generous gift from C.M. Rice, Rockefeller University, NY, USA, was seeded and maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 1% penicillin/streptomycin, 1% non-essential amino acids, and 10% fetal bovine serum (FBS) in a humidified incubator atmosphere of 5% CO2 *v/v* in air, at 37 °C, all purchased from Gibco TM. The cells were incubated and subcultured for at least three passages before transfecting them with HCV RNA, which was prepared as follows.

Plasmid preparation

Two plasmids, JFH1 (genotype 2a) full length and 4a (ED43/C-NS2/NS5A), were used in the assay with generously donated materials from the University of Copenhagen, Denmark, Department of Infectious Diseases and Clinical Research Center.

Digestion of plasmid DNA and RNA synthesis

Plasmids about 40 μg each used for *in vitro* transcription were first digested by the restriction enzyme Xba1 (Fermentas, Germany). Plasmid linearization was done using 3 μL from the plasmid and 1.2 μL from xbal, 5 μL 10x buffer and the complete volume was 50 μL with H_2O. The volume was incubated at 37 °C for 1 h. Validation of complete digestion was done through agarose gel electrophoresis. To create blunt ends in digested plasmids, a mung bean nuclease was used and followed by incubation period of 30 minutes at 37 °C. A mixture of 25 μL phenol, 24 μL chloroform and 1μL isoamyl alcohol was used for DNA purification. Centrifugation at 14000 rpm for 5 minutes was carried out for precipitation and separation of aqueous layer. After adding an equal volume of chloroform to the aqueous phase and transfer into a new tube, it was mixed and centrifuged at 14000 rpm for 5 minutes. Then, twice the volume of cold ethanol was added to the aqueous phase in a new tube, followed by centrifugation at 14000 rpm for 10 minutes. The pellets were washed in 500 μL of 70% cold ethanol, then centrifuged at 14000 rpm for 10 minutes. The pellets were air dried for 5-10 min after ethanol was discarded. RNase-free water (10 μL) was used to dissolve the pellet.

For RNA synthesis using MEGAscript T7 (Ambion), a mixture consisted of 2 μL of transcription buffer (5X), 2 μL of 10 mM ATP/GTP/CTP/UTP Mixture, 1 μg of linearized template DNA, 0.5 μL of (20U) RNase inhibitor, 2 μL of (30U) T7 Polymerase, and RNase-free water up to 25 μL for final reaction mixture. The final mixture was kept for a 2-h incubation period at 37 ˚C. After incubation, 2U of DNase were added to the final mixture and incubated at 37 ˚C for 15 minutes, after which 15 μL of ammonium acetate was added. A nanodrop (Quawell, UV-Vis spectrophotometer Q5000) was used to determine final concentration of synthesized RNA.

HCV RNA transfection

Huh7.5 cells ($0.7x10^6$) were incubated for 24 h, washed twice with PBS, then transfected with HCV RNA using a mixture of Opti-Mem serum-reduced medium (Thermo Fischer Scientific) and lipofectamine 2000 reagent (Invitrogen). The cells were then incubated for 24 h, after which the medium was replaced with DMEM supplemented with 1% non-essential amino acids and 10% FBS.

Concentration of the virus

After incubation period of 48 h, culture filtrates were collected every 2 days over the course of 7 days, and cells were preserved at last. Collected culture filtrates were concentrated using Amicon Ultra 15 centrifugal filter units at -4 ˚C for 30 minutes. The concentrated virus was preserved at -80 ˚C for long-term preservation[9,16].

Infection of the cells

Cells were seeded at an exponential growth phase at $35x10^4$ cells *per* well in a 6-well plate for 24 h to allow development of an approximately 90% cell monolayer. RNAs were diluted in serum-free medium to the optimum concentrations required for infection and incubated for 2 h at 37 ˚C in humidified CO2. After incubation, each well overall volume continued to increase to 2 mL with complete DMEM supplemented with 5% FBS, and was incubated for 48 h for viral detection[2].

Viral load analysis by RT-PCR

The HCV quantitative RT-PCR kit (Qiagen, USA) was used to determine the RNA copy number of hepatitis C virus (HCV) in Huh7.5 cells. HCV RNA extracted from infected cells is a direct reflection of cell infection. After incubation, viral RNA was isolated from Huh7.5 cells using the RNeasy Plus Mini Kit (Qiagen, Germany). HCV RNA load was detected using the Qiagen HCV RT-PCR Kit (USA) according to the manufacturer's instructions. The RT-PCR program included 15 minutes at 50 ˚C (for cDNA synthesis), 10 minutes at 95 ˚C (for initial denaturation) for 45 cycles, with denaturation at 95 ˚C for 15 seconds, 20 seconds of annealing at 55 ˚C, and 15 seconds of extension at 72 ˚C[17].

Immunostaining of Huh7.5 cells after infection

Huh7.5 were seeded in an 8-well Corning Biocoat collagen culture slide to reach confluence after 24 h of incubation in a humidified cell incubator with 5% CO2 and 95% air at 37 ˚C using DMEM supplemented with 1% penicillin-streptomycin, 1% non-essential amino acids, and 10% FBS. After incubation, a mixture of concentrated virus with two different genotypes (2a and 4a) over the cells and control cells without virus was incubated for 2 h. The wells of cultured slides were washed from treatments, and DMEM supplemented with 1% penicillin-streptomycin, 1% non-essential amino acids and 10% FBS was added to cover each well. The slides were then incubated at 37 ˚C for 48 h. After incubation, the cells were washed with PBS. The fixation of cells was done using 4% paraformaldehyde in PBS for 1 h at room temperature, followed by 15 minutes of permeabilization in 0.1% Triton X-100 in PBS. Blocking buffer mixtures from bovine serum albumin, FBS and PBS were added for 1 h at room temperature. Primary antibodies (anti HCV-NS5a antibody) were diluted in PBS and incubated with cells for 2 h at room temperature. After three repeated washing cycles in PBS, Alexa Fluor 488 goat anti-mouse IgG (Invitrogen) was added to cells at a 1:1000 dilution for 1 h at room temperature in a dark room. After staining, the slides were washed in PBS and mounted in ProLong Antifade. The slides were examined under a Zeiss Axiostar fluorescence microscope[18].

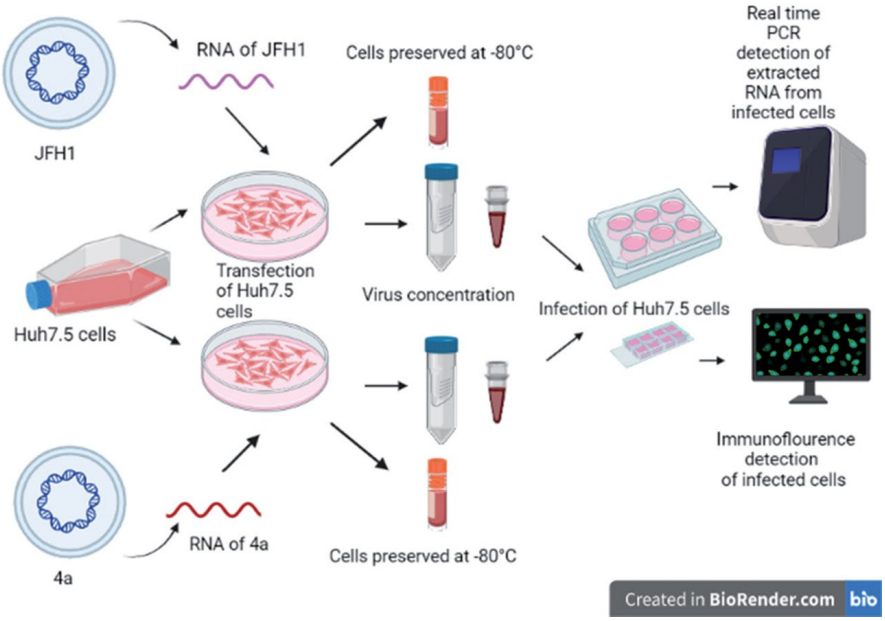

Fig. 1. Summary of the protocol for HCV cell culture system.

Results and Discussion

Transfection of Huh7.5 cells

The production of HCV infectious particles was established with the first step of delivery of genomic JFH1 and 4a RNAs in Huh7.5 cells. Transfected cells were incubated, and at two days post-transfection, the supernatants collected from infectious HCV cell culture were centrifuged to be concentrated in small volumes, and then preserved to be used in infection. All steps of HCV cell culture system are illustrated in Figure 1 using https://app.biorender.com. Initiation of HCV RNA replication in Huh7, especially 7.5, is more permissive, also providing a powerful substrate for HCV genetic and biochemical studies. Transfected Huh7.5 cells were passaged when they reached confluence. Huh7.5 cells were efficiently used for HCV viral replication[19]. A study[20] illustrated a replicon containing the highly adaptive NS5A S2204I mutation and determined transduction efficacy based on the number of cells used for electroporation based on a more sensitive analysis that showed survival of >75% of the cells after the infection procedure harboring HCV RNAs. The development of HCV cell culture systems provides a great potential in various mechanisms of actions in viral life cycle. The successful process of transfecting Huh7.5 cells is the core through which the indicative viral infection with higher viral load was achieved.

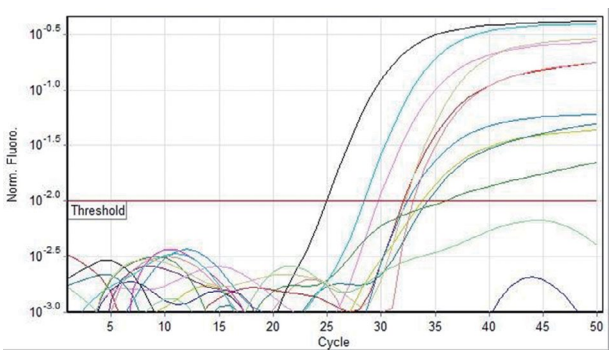

Fig. 2. Real-time polymerase chain reaction (RT-PCR) curve of both standard and HCV genotype 2 and 4a concentration. RT-PCR curve indicates three standards from 125×103 to 12.5×105 copies/mL and sample genotypes 2 and 4a with 32,500 and 107,375 copies/mL, respectively.

Table 1. Real-time polymerase chain reaction indicates three standards from 125×03 to 12.5×105 copies/mL (column 1): sample genotypes 2 and 4a and standard arrangements; Ct (column 2): Ct value of sample genotypes 2 and 4a and standards; given concentrations (copies/mL) (column 3): quantification standard concentrations; calc. concentration (copies/mL) (column 4): concentrations of the samples and standards that were measured by Rotor Gene.

	Ct	Given concentration (copies/mL)	Calc. concentration (copies/mL)
Genotype 2	34.30		32,500
Genotype 4	32.41		107,375
Standard 1	32.24	125,000	119,955
Standard 2	28.41	1,250,000	1,357,345
Standard 3	24.96	12,500,000	11,995,541

Viral load analysis by RT-PCR

The HCV RNA extracted from infected Huh7.5 was determined with RT-QPCR and indicated that the two genotypes (2 and 4) resulted in 32,500 and 107,375 copies/mL, respectively (Fig. 2) detected from the standard in Table 1. Huh7.5 cells infected with concentrated progeny viruses could effectively infect naïve Huh7.5 cells. The two genotypes, 2 and 4, had different outcomes of 32,500 and 107,375 copies/mL, indicating that genotype 4 could infect more cells over the same time period compared to genotype 2. The J6/JFH control virus delayed the production of viruses when compared to 4a/JFH1. However, the infection spread and increased HCV RNA in addition to the infectivity titers achieved in serial passages[8]. This is illustrated in Figure 2.

Detection of immunofluorescence for infected Huh7.5 cells

The infected cells with JFH1 (genotype 2a) and 4a (ED43/C-NS2/NS5A) indicated a positive result when compared to stained untreated cells (Fig. 3). The presence of green fluorescence showed in the cells as a positive result of viral entry when compared to untreated cells. Immunofluorescence is one of the main protocols that provides the target specificity in HCV 7.5 infected cells.

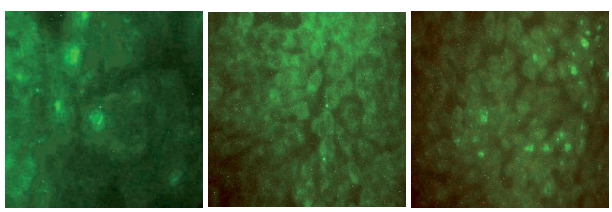

Fig. 3. Immunostaining indicates control cells without viral treatment, cells infected with RNAs of JFH1 (genotype 2a) and 4a (ED43/C-NS2/NS5A).

Future prospective of the study

The HCV cell culture systems allow research of the virus life cycle and antiviral targets in processes such as virus entry, processing, assembly, etc., showing great potential in advancing antiviral pharmacotherapy and vaccine research. So, it is concluded that cell culture systems play a great role in biotechnological and applied research for both testing the efficiency of HCV vaccines and expanding knowledge about cellular, molecular, and biological aspects of the virus life cycle, as well as the evaluation of antiviral drugs against HCV genotypes 2a and 4a.

Conclusion

There are many studies that perform HCV antiviral research from different aspects including viral inhibition by cell pretreatment, viral inhibition by virus pretreatment, and viral replication inhibition assay. In viral inhibition by cell pretreatment, extracts or compounds were first screened for their antiviral effect throughout the adsorption phase by means of blocking cellular receptors. This had been achieved by preincubation of these extracts or compounds before the cells were infected with the test virus. Regarding viral inhibition by virus pretreatment, the extracts and

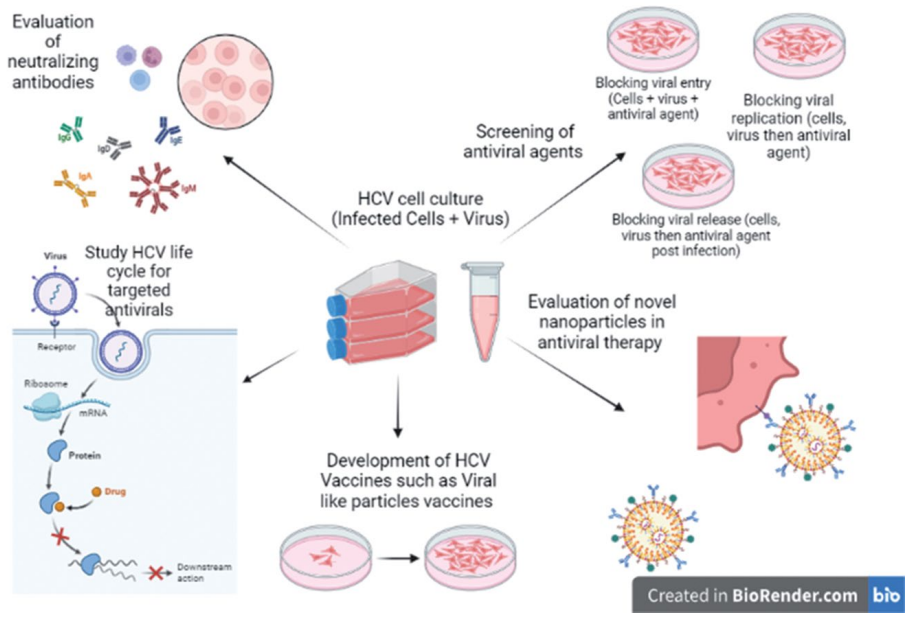

Fig. 4. Application of optimized HCV cell culture system in antiviral research.

compounds combat viral infection *via* blockage of viral entry into the cells or by means of viral inactivation. The potential to disrupt the viral replication process and interfere with viral replication with different efficiencies was tested through viral replication[21].

Different contributions of HCV cell culture system are important in facing the challenges of the HCV therapy research. The application of different assays in the same protocol through using various targets of viral life cycle may have contributed to overcoming challenges such as drug interactions in a significant number of HCV patients during the last decade. Also, the natural progress of resistant variant-associated differences in the viral life cycle added significant value to HCV cell culture systems. The higher titer produced from infectious virions in HCV cell culture system provides an accurate determination of the effectiveness of antiviral compounds[22].

Optimization of HCV system is one of the main factors that contribute to using HCV in antiviral research. The role of HCV cell culture system provides evaluation of using small molecules and antibodies as antiviral therapy. It also contributes to the possibility of screening the broad-spectrum antiviral agents. Screening of antivirals could be developed during studying the viral life cycle from entry to release. Figure 4 illustrates the application of optimized HCV cell culture system in antiviral research.

The study illustrates the optimization parameters for the HCV cell culture system. The overall process including transfection and infection depends on the viability of Huh7.5 cells. Also, increasing the viral load through concentration of the virus leads to successful infection. At last, the process of making aliquots from the concentrated virus is important to avoid viral degradation through freezing and thawing processes. The aim of the study was to highlight the use of HCV cell culture systems as a rapid tool to study the antiviral activity of different sources. The preparation of an efficient infective virus through an HCV cell culture system using two different genotypes could be used as a screening tool for active antiviral substances. Also, the culture system contributes to determining the mode of action of each antiviral agent by using pre-infection, co-infection, and post-infection assays.

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Sažetak

OPTIMIZACIJA SUSTAVA HCV KULTURA STANICA ZA EVALUACIJU ANTIVIRUSNIH LIJEKOVA PROTIV HCV GENOTIPOVA 2a I 4a

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Procjena infekcije virusom hepatitisa C (HCV) u staničnoj kulturi važna je za razvoj novih antivirusnih lijekova i za razumijevanje životnog ciklusa virusa, kao i za proučavanje korelacije među genotipovima koji se šire diljem svijeta. Razvoj sustava HCV stanične kulture predstavlja veliku prednost jer omogućava rast virusa u staničnoj kulturi, što otvara mogućnost otkrivanja potencijalnih lijekova i cjepiva. Cilj ovog istraživanja bio je prikazati korake infekcije HCV-om u sustavu stanične kulture kao pokazatelje za učinkovitost snažnijih antivirusnih spojeva. Dva plazmida, JFH1 (genotip 2a) pune duljine i 4a (ED43/C-NS2/NS5A) podvrgnuta su *in vitro* transkripciji radi sinteze HCV RNA, nakon čega je uslijedila transfekcija Huh7.5 stanica s dobivenom RNA iz svakog genotipa kako bi se generirao virus. Kasnije su Huh7.5 stanice zaražene repliciranim virusom rezultirale stvaranjem stanica zaraženih HCV-om. Imunobojanje zaraženih stanica pokazalo je fluorescenciju zelene boje, što ukazuje na pozitivnu infekciju oba genotipa, 2a i 4a. Usto, provedena je i *real-time* lančana reakcija polimerazom radi mjerenja virusnog opterećenja iz ekstrahiranih stanica prethodno zaraženih s RNA genotipova 2 i 4, što je rezultiralo s 32.500, odnosno 107.375 kopija/mL. Zaključno, uvođenje sustava HCV stanične kulture predstavlja prikladnu metodu za procjenu aktivnih antivirusnih lijekova protiv HCV genotipova 2a i 4a korištenjem kvalitativnih i kvantitativnih tehnika u razlikovanju učinkovitosti antivirusnih lijekova.

Ključne riječi: *Hepatitis C virus; Infekcija; Huh7.5; JFH1; Genotip 4*