

Construction of the Vero cell culture system that can produce infectious HCV particles

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Abstract The hepatitis C virus is a major cause of chronic liver disease worldwide. Lack of culture system supporting virus production has been one of the major impediments in HCV research and vaccine development. Here, we use a HCV (1b) full-length cDNA clone that replicates and produces integrated and infectious virus particles in cultured Vero cells. Evidence shows that the replication of virus particles is robust, producing over 10^8 copies of positive RNA per milliliter of the culture cells within 48 h. Sucrose density gradient centrifugation of the cell lysate reveals that the HCV virions have a density of about 1.17 g/ml and a spherical morphology with an average diameter of about 55 nm. Secreted virus is infectious for Huh7 cells and can be neutralized by CD81- and E2-specific antibodies. This system establishes a powerful framework for studying the virus life cycle and developing vaccine research.

Keywords Hepatitis C virus · Vero cell culture · Full-length cDNA clone · Infectivity · Huh7

Introduction

Hepatitis C Virus (HCV) is a major cause of chronic liver disease worldwide which progresses to acute and chronic hepatitis and hepatocellular carcinoma [1]. HCV

is an enveloped, positive-sense RNA (~9.6 kb) virus of the family *Flaviviridae*. It has a central ORF flanked by the 5'- and 3'-noncoding regions. The ORF encodes one large polypeptide that can produce both the structural proteins (core and glycoprotein E1 and E2) and the nonstructural proteins (p7 through NS5B). Current drug therapies are poorly tolerated and barely effective in patients; there is no vaccine for HCV [2, 3]. In 2005, scientists in Japan and U.S.A. built up an in vitro culture system of HCV [4–6], which has significantly enhanced our understanding of viral life cycle. Nevertheless, it does not contribute to the preventive techniques on effective therapeutics for HCV. A major impediment in HCV prevention and therapy is the limitation of anti-HCV medication in HCV therapy. A full-virion vaccine as a conventional method plays important role in virus prevention.

HCV is divided into six genotypes (I, II, III, IV, V and VI) according to their homogeneity, and there are a number of subgenotypes in a single genotype. The multiformity of HCV genotypes affords unique opportunity for us in investigating its chorology and epidemiology, but it also poses challenge in virus prevention. So far, research on HCV vaccine has focused on two aspects, one being the recombinant polypeptide vaccine based on the epitope of virus proteins and the other being DNA based vaccine. Some of these vaccines have been tested on animals and proven effectively to a certain extent. However, due to the variability of the RNA virus and the lack of the cell culture system to support the virus production, there is yet a big gap to bridge between the research and application of the vaccines [7–9].

In this study, we report the construction of a highly efficient in vitro infectious system based on Vero cell line and the pHCV-WHU-1 consensus clone (genotype 1b)

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without adaptive mutations. This system produced high levels of HCV genome ($>10^8$ copies/ml) with the aid of T7 polymerase provided by recombinant vaccinia virus vTF7-3. HCV particles obtained from the cultured media were filterable and infectious with natural Huh7 cells. Vero cell line is safe for vaccine production and HCV genotype 1 is the major genotype of human infections worldwide and is the most intractable type. Therefore, our system provides an opportunity to further the study of the HCV life cycle and offers an alternative route of HCV vaccine preparation.

Materials and methods

Plasmid construction

The cloning of the HCV full-length genome and construction of the plasmid were accomplished previously by Li [10]. Briefly, total RNA was extracted from sera of the patient infected by acute hepatitis C by using RNA extraction kit. Full-length HCV genome was generated from viral RNA extractions by RT-PCR. In this process, six pairs of primers (Fig. 1a) were designed according to the HCV genome reported by Aizaki [11] and T7 promoter was added in the primer 6 lying in the upstream of 5'-UTR. pHCV-WHU-1 plasmid was generated by inserting a *HindIII*–*SpeI* fragment containing the full-length HCV genome into *HindIII*–*SpeI* sites of pFK-1 which was generated from pBR322 without tetracycline gene (Fig. 1b). The recombinant plasmid pHCV-WHU-1 was used to transfect cells.

Cell culture

Vero cells were maintained at 37°C in 5% CO₂ in Eagle's Minimum Essential Medium supplemented with 10% fetal bovine serum (Gibco). Huh7 cells were maintained at 37°C in 5% CO₂ in F-12K Medium supplemented with 10% fetal bovine serum.

Cell transfection

First, 2×10^5 Vero cells were infected with vTF7-3 (10^{10} TCID₅₀, 2,000-fold dilution) for 2 h. Then vTF7-3 was removed and 1.6 µg pHCV-WHU-1 was transfected into Vero cells with Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's protocol. For the control experiment, cells were transfected with pHCV-WHU-1 only. Transfection efficiency was evaluated by the synthesis of positive-strand RNA by RT-PCR.

RNA extraction, reverse transcription-PCR (RT-PCR) and fluorescent quantitative PCR

Transfected Vero cells and infected Huh7 cells were harvested at different times. RNA was extracted by Trizol reagent (Invitrogen) based on the protocol instructed by the manufacturer. Total RNA was treated with DNase I (37°C 30 min) to remove any plasmid DNA. Reverse transcription and PCR of the positive-strand HCV RNA was performed as previously described [12]. Fluorescent quantitative PCR was performed according to the manufacturer's protocol (Hepatitis C virus fluorescence polymerase chain reaction diagnostic kit was purchased from Daangene Co. Ltd, China).

Actinomycin D inhibits the activity of T7 polymerase experiment

Actinomycin D (Act.D) (Sigma) with different concentration: 0, 0.2, 1, 2 µg/ml were added into the 4–8 h transfected Vero cells in a 12-well plate and cultured for another 24 h at 37°C. The efficiency of the inhibition of the Act.D was monitored by fluorescent quantitative PCR and strand-specific RT-PCR.

Detection of the negative-strand RNA of HCV

We used both strand specific RT-PCR and nested PCR to detect the negative-strand RNA of HCV. RNA was extracted from the transfected Vero cells and the infected Huh7 cells by Trizol reagent. Then we first used the RTP: 5'-TCA TGGTGGCGAATAACGCCACAGGACGTTAAG-3' to amplify the cDNA of HCV, and used a pair of specific primers 5'-ATGTACCCCATGAGGTCCGC-3' and 5'-TCA TGGTGGCGAATAA-3' to amplify a 375 bp fragment. Another pair of primers 5'-CAGATCGTTGGTGGAG-3' and 5'-TGTGAGGGTATCGATGAC-3' was used to amplify the target fragment 297 bp, which was located in the 5'-untranslated region of HCV.

Western blot analysis

Western blot analysis was performed according to the reference described previously [13]. Cells were washed once with PBS (0.1 M) and detached from the plate by treating 0.05% trypsin–0.02% EDTA. Cells contained in a small aliquot of the suspension were counted. Aliquots of cell lysates corresponding to 2×10^6 cells were analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (12% or 15% polyacrylamide) and transferred to a

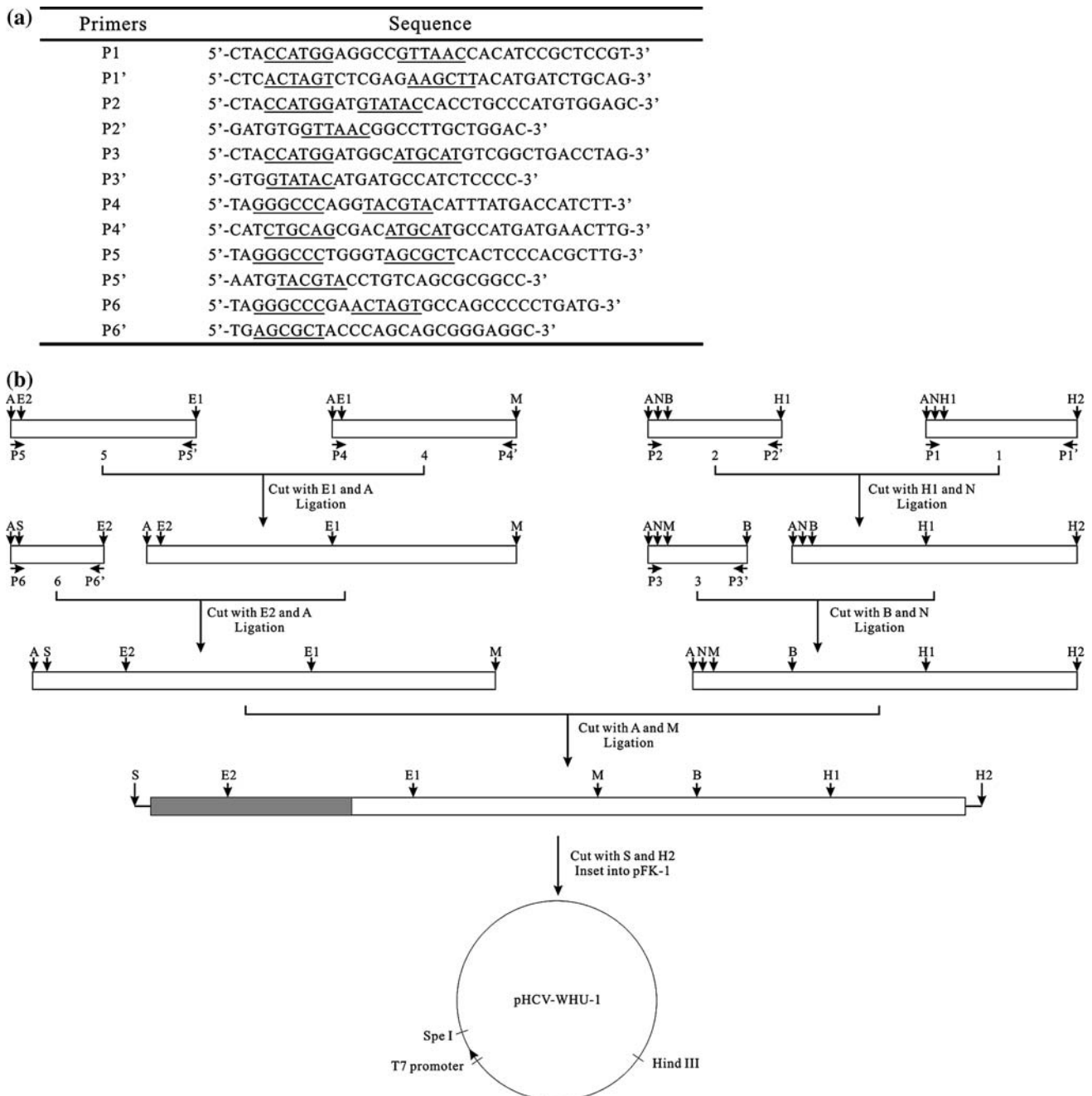


Fig. 1 Construction of the HCV full-length cDNA clone. (a) Primers for HCV full-length cDNA clone. Enzyme sites in the sequences are underlined. (b) Strategy for constructing a full-length cDNA clone of HCV. The locations of primers are shown. Arrows point the enzyme

sites. A: *Apa*I; B: *Bst*1107I; E1: *Eco*105I; E2: *Eco*47III; H1: *Hpa*I; H2: *Hind*III; M: *Mph*1103I; N: *Nco*I; S: *Spe*I. Shaded and open boxes indicate structural and nonstructural proteins, respectively. UTRs are indicated by bars

polyvinylidene difluoride membrane. The primary polyclonal antibodies (rabbit anti-Core, E1, E2 and NS5B proteins of HCV) were kindly presented by Prof. Ye Linbo (Wuhan University). The secondary antibody horse anti-rabbit IgG conjugated with alkaline phosphatase was offered by Proteintech Group, Inc. NBT-BCIP was used as substrate. GAPDH was used as system control and antibody to GAPDH was offered by Proteintech Group, Inc.

Sucrose gradient density centrifugation

A total of 4×10^7 Vero cells were harvested at 68 h after transfected with pHCV-WHU-1, washed by PBS (0.1 M) once and suspended in water. Cells were treated with Ultrasonic Cell Disrupter System on ice twice and then the whole cell lysates were centrifuged at 3,000 r/min for 10 min at 4°C once after the disposal. The suspension

was collected and passed through a 0.22- μm filter, then centrifuged at 35,000 r/min for 3 h at 4°C in Ti70 tubes (Beckman Coulter) after treated with Homogenizer on ice once. Subsequently, the deposition was suspended in water and applied onto a 10–60% sucrose gradient (9.5-ml volume) in SW40 tubes (Beckman Coulter) and centrifuged at 100,000g for 16 h at 4°C. We collected every 0.5 ml fraction from the top of the gradient. OD₂₆₀ of the fractions were tested by BioPhtometer (Eppendorf). And the fractions with higher OD₂₆₀ were selected and suspended in water to wash the sucrose by centrifuging at 35,000 r/min for 3 h at 4°C. The pellets were suspended in 200 μl water.

Electron microscopy observation of HCV particles

A total of 2×10^6 Vero cells were harvested at 68 h after transfected with pHCV-WHU-1, and 2×10^6 Huh7 cells were harvested at 4 d after infected with HCV virions, respectively. Harvested cells were washed by PBS (0.1 M) once and fixed by 2.5% glutaraldehyde at 4°C overnight. Sample preparation according to the proceeding routinely: dehydration, embedding, slice up and dyeing.

For negatively stained observation, suspension from sucrose gradient density centrifugation assay was put on formavar/carbon-coated copper grids. The grids were negatively stained with 2% phosphotungstic acid (PTA, pH 6.5) and observed under a transmission electron microscope (Hitachi, HB8100).

Immunoelectron microscopy was performed according to the protocol described by other researchers [14, 15]. Mouse anti-HCV E2 monoclonal antibody (Virostat, USA) was used as a primary antibody. Goat anti-mouse immunoglobulin colloidal gold particles (10 nm in diameter, purchased from Beijing Biosynthesis Biotechnology Co. Ltd., China) was utilized as a secondary antibody.

Infection experiment

Permissive cell line Huh7 (2×10^5) was inoculated with the HCV particles suspension (concentration of 10^7 copies RNA/ml) at 37°C for 12 h. The medium was changed with F-12K Medium containing 5% fetal bovine serum and cultured at 37°C for a certain amount of time with 5% CO₂. HCV particles used in the experiment were harvested and purified by the sucrose gradient density centrifugation. The infectivity was estimated by fluorescent quantitative PCR and western blot analysis.

Blockade of HCV infection by anti-E2 antibody

Mouse monoclonal anti-E2 antibody (ViroStat) at 100 $\mu\text{g}/\text{ml}$ was supplemented with pure HCV particles suspension overnight at 4°C. Then Huh7 (2×10^5) was inoculated with this suspension as described above. The infection efficiency was monitored 6 d post infection by RT-PCR and Western blot analysis.

Blockade of HCV infection by CD81 antibody

Mouse monoclonal anti-human CD81 antibody (Protein-tech Group, Inc.) at 1 mg/ml was serially diluted (1:2,000, 1:200 and 1:20) and preincubated in a volume of 500 μl with 2×10^5 Huh7 cells in a 12-well plate for 2 h at 37°C. Cells were subsequently inoculated with infectious HCV particles at a concentration of 10^7 copies RNA/ml for 12 h at 37°C. The infection efficiency of the presence of antibodies was monitored 3 d post infection by fluorescent quantitative PCR.

Results

Production of HCV particles

HCV RNA production in the transfected Vero cells

HCV positive-strand RNA was detected by RT-PCR in the Vero cells both infected with vTF7-3 and transfected with pHCV-WHU-1 at 12, 24 and 48 h post transfection (Fig. 2a), indicating the synthesis of positive-strand RNA in this cell culture system. Sequence analysis showed that there was 100% match between the 311 bp target sequence and the HCV 5'-UTR (data not shown). In contrast, target fragment was not acquired in the controls. This indicated that there is no production of positive-strand RNA without the aid of T7 polymerase offered by vTF7-3.

The negative-strand RNA is an important intermediate during the replication process of RNA viruses. To avoid the possibility that negative-strand RNA could have been produced from the transfected cells due to activity of T7 polymerase, cells were treated with Act.D. Act.D is known to inhibit DNA-dependent RNA polymerase. Detection of negative-strand RNA in the presence of Act.D is an indication of HCV RNA synthesis. After culturing for 24 h, we detected the positive- and negative-strand HCV RNA, respectively. As shown in Fig. 2b, intracellular HCV RNA levels were reduced in a dose-dependent manner with a 33-fold reduction at 2 $\mu\text{g}/\text{ml}$ Act.D as compared with the controls. And the RNA levels were decreased compared with before Act.D was added. On the other hand, the

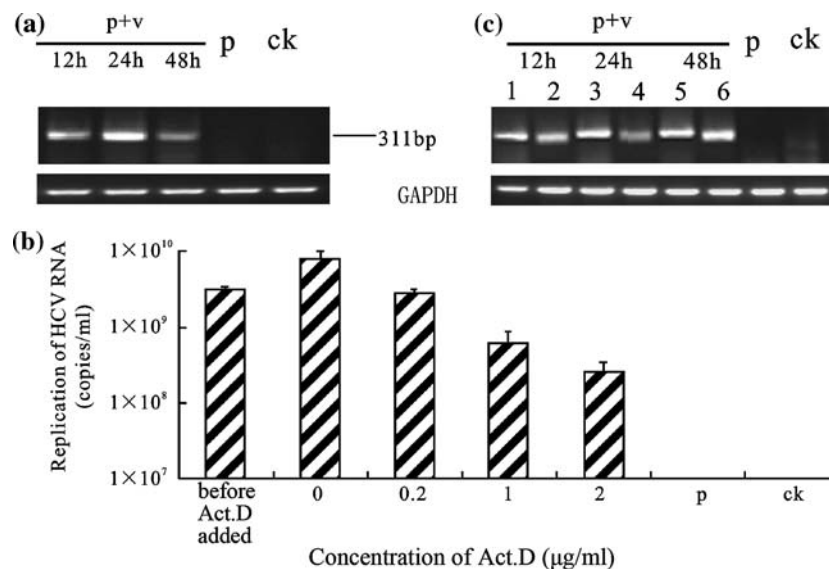


Fig. 2 Detection of HCV RNA in the transfected Vero cells. (a) Detection of positive-strand RNA in the transfected Vero cells by RT-PCR. (b) Detection of positive-strand RNA in the transfected Vero cells treated with Act.D by fluorescent quantitative PCR. The data of the column are the average of two detections. The error bars are at the top of the columns. (c) Detection of negative-strand RNA in the

transfected Vero cells treated with Act.D (2 µg/ml) by strand-specific RT-PCR and nested PCR. 1, 3, 5: 375 bp; 2, 4, 6: 297 bp. p + v: Vero cells both infected with vTF7-3 and transfected with pHCV-WHU-1; p: Vero cells transfected with pHCV-WHU-1 only; ck: Vero cells neither infected with vTF7-3 nor transfected with pHCV-WHU-1. GAPDH played as system control

negative-strand HCV RNA was detected in the transfected Vero cells after the addition of Act.D, as shown in Fig. 2c. Furthermore, sequence analysis showed that the target fragment 297 bp matched with the HCV 5'-UTR (data not shown). Therefore, the detection of negative-sense HCV RNA demonstrated the genome replication.

The kinetic characteristics of HCV genome in the transfected Vero cells

Fluorescent quantitative PCR assay revealed that the copies of HCV RNA decreased with the elapse of time in the transfected Vero cells. The titer peaked at 12 h post transfection, reaching $(4.34 \pm 1.04) \times 10^9$ copies/ml and reduced to $(1.15 \pm 0.76) \times 10^9$ copies/ml at 48 h. With the elapse of the culture time, the copies of HCV RNA presented degressive tendency with the titer from $\sim 4 \times 10^9$ to $\sim 1 \times 10^8$ copies/ml (Fig. 3a). In the control cells, the detected numerical value was less than 10^3 copies/ml (the fluorescent background was from 10 to 1,000 when reflecting on the numerical value), so we regarded it as a negative one. Therefore, the plasmid pHCV-WHU-1 can transcribe and replicate HCV genome efficiently with the aid of T7 polymerase offered by vTF7-3 in the transfected Vero cells. Furthermore, the concentration of HCV RNA in the cultured media was more than 10^7 copies/ml, which is roughly 100 times in amount compared with that

in the infected serum. The large number of viral genomic RNA is potential for the preparation of vaccine.

HCV protein expression in the transfected Vero cells

Western blot analysis detected the expression of HCV protein in the transfected Vero cells. Results shown in Fig. 3b demonstrated that transfected Vero cells produced HCV structural (Core 22 kD, E1 35 kD, E2 72 kD) and non-structural (NS5B 68 kD) proteins efficiently. We concluded that the amount of protein expression at 68 h post transfection was much more than at 48 h in the Vero cells. Simultaneously, HCV proteins could not be detected in the controls, which indicated that HCV RNA production with the aid of vTF7-3 is the precondition of proteins expression. The viral protein produced from the cultured Vero system has the same immunocompetence as that of the crude virus, since it can combine with the corresponding antibodies.

HCV virions production and electron microscopic observation

We successfully observed HCV particles in the Vero cells transfected with pHCV-WHU-1 (Fig. 4a), the virions are all located in the cytoplasm with a diameter about 55 nm. In addition, the HCV particles and vTF7-3 particles were also

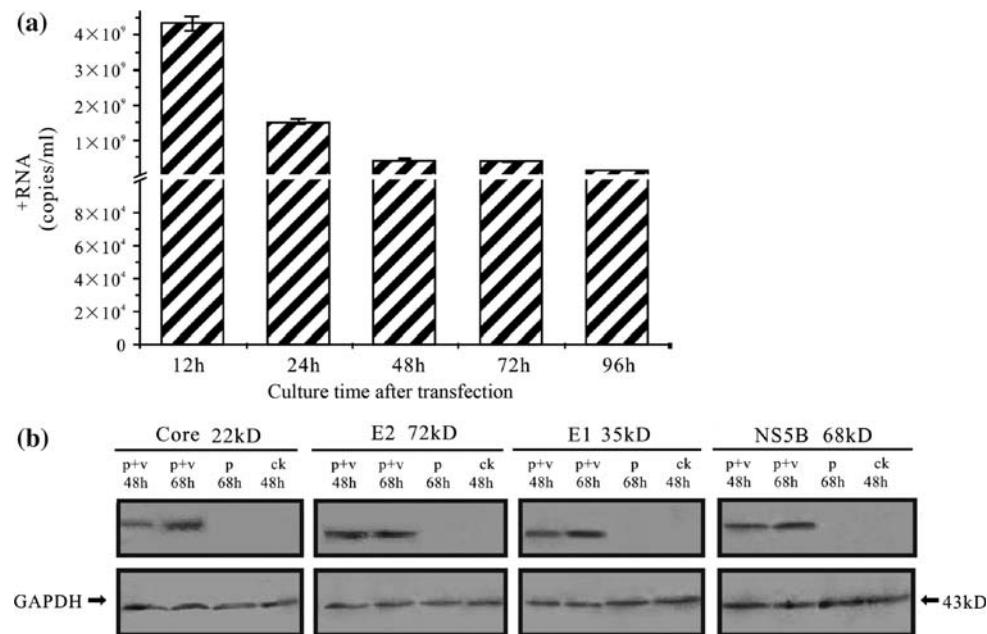


Fig. 3 Kinetic curve of HCV RNA and proteins expression in the transfected Vero cells. **(a)** Kinetic curve about the decrease of HCV genome in the transfected Vero cells. The data of the column are the average of three detections. The error bars are at the top of the columns. **(b)** Western blot analysis of Vero cells for HCV proteins Core, E1, E2 and NS5B. Vero cells transfected with pHCV-WHU-1

only and Vero cells treated with nothing were played with negative controls. p + v: Vero cells both infected with vTF7-3 and transfected with pHCV-WHU-1; p: Vero cells transfected with pHCV-WHU-1 only; ck: Vero cells neither infected with vTF7-3 nor transfected with pHCV-WHU-1. GAPDH played as system control

found in the same cell, which indicated that the vTF7-3 plays an important role in the production of HCV particles. Furthermore, we also observed HCV particles were released from the cell membrane, suggesting that Vero cell line is producing intact and infectious HCV particles (Fig. 4b).

To assess the capability of HCV particles production, Vero cells transfected with pHCV-WHU-1 were subject to sucrose density gradient centrifugation. The fractions were analyzed for OD₂₆₀ (Fig. 4c). Fractions (from 17 to 22) with higher OD₂₆₀ (>3.0) were selected to wash sucrose, followed by centrifuging. By using conventional negative contrast electron microscopy, we observed spherical HCV-like particles 55 nm in diameter averagely (Fig. 4d) in the purified virus suspension from fraction 17, which is compatible with the predicted size of HCV [6, 14, 16–18]. The size of the particles was homogeneous and could be labeled by anti-E2 monoantibodies by using electronmicroscopy immunoglobulin colloidal gold particles labeling (Fig. 4e). These results confirmed that virion was assembled by HCV proteins and genome in the transfected Vero cells. The observed virions have the same physical characteristics as those of the wild ones, suggesting that HCV particles from the Vero cell culture system will have the same structure and activity as that of the wild virus. At the same time, we observed the vaccinia virus vTF7-3 in the suspension from the fraction 20 (data not shown) but nothing in the other four fractions.

Infectivity of HCV particles

HCV RNA production in the infected Huh7 cells

To ensure that the HCV virions produced from the Vero culture system were infectious, we infected the permissive cell line Huh7 with the purified HCV particles obtained from the sucrose density gradient centrifugation. As shown in Fig. 5a, we detected the presence of positive-strand RNA in the infected Huh7 cells at 2, 4 and 6 d post infection without passage. The amplified fragment was consistent with what we designed. This indicated that HCV particles produced from cultured Vero cells were infectious and the positive-strand RNA can be synthesized efficiently.

Similarly, specific detection of the intermediate, negative-strand RNA, was crucial to proving the active replication of HCV in the infected cells. We detected the negative-strand RNA by strand-specific RT-PCR at 2, 4 and 6 d post infection without passage, and the concentration of the PCR resultant increased with the elapse of culture time, suggesting that HCV virions could self-replicate efficiently in Huh7 cells and form the negative-stranded RNA intermediate (Fig. 5b). The size of the fragment amplified completely matched with the HCV 5'-UTR.

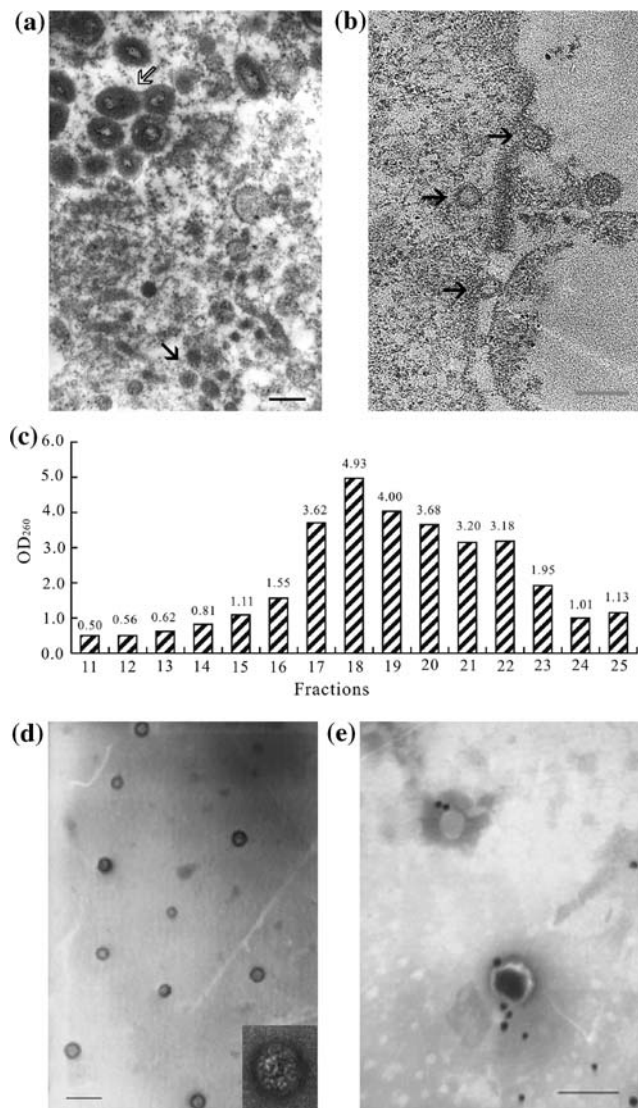


Fig. 4 Electron microscopic observation of the HCV particles from the transfected Vero cells. (a) Electron microscopy observation of HCV and vTF7-3 particles in transfected Vero cells. (b) HCV particles release from the Vero cell membrane. (c) Detection of OD₂₆₀ of the fractions from sucrose density gradient centrifugation. The OD₂₆₀ numerical value was at the top of the columns separately. Fractions from 1 to 10 were omitted because their OD₂₆₀ were less than 0.50. (d) Negative contrast electron microscopic observation of HCV. Virus-like particles of HCV about 55 nm in diameter were found, inset is the magnified HCV particle. (e) Immunoelectron microscopic observation of HCV. The immunogold particles were 10 nm in diameter. \uparrow : vTF7-3 particles; \uparrow : HCV particles. Scale bar represents 100 nm

The kinetic characteristics of HCV genome in the infected Huh7 cells

Fluorescent quantitative PCR assay revealed that the copies of HCV RNA increased with the elapse of time in the infected Huh7 cells. The copies peaked at 6 d post infection, reaching $(4.72 \pm 1.42) \times 10^4$ copies/ μ g of total RNA

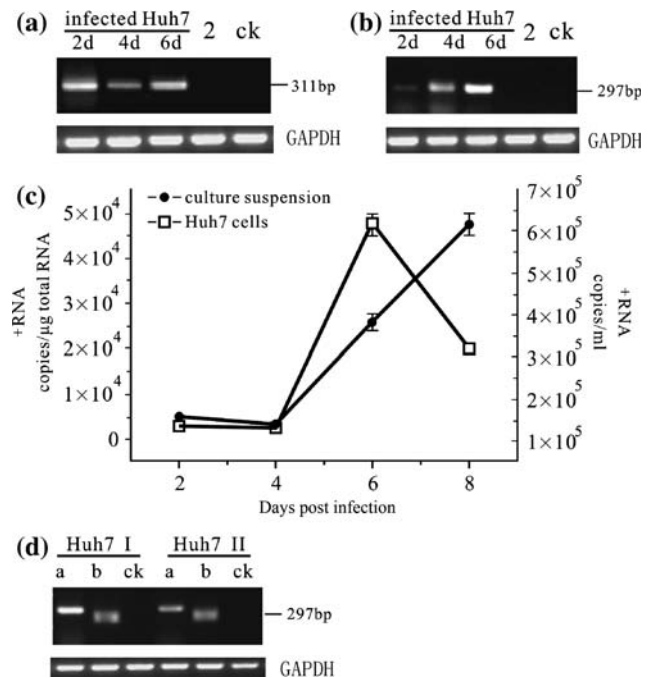


Fig. 5 Detection of HCV RNA in the infected Huh7 cells. (a) Detection of positive-strand RNA in the Huh7 cells by RT-PCR. (b) Detection of negative-strand RNA in the Huh7 cells by strand-specific RT-PCR and nested PCR. 2: Huh7 cells infected by HCV virions which were supplemented with anti-E2 monoclonal antibody overnight; ck: natural Huh7 cells. Days post infection was at the top of the picture. (c) Kinetic curve of the HCV genome replication in the infected Huh7 cells. The data of the curve represent the average of two experiments with error bars. Square: infected Huh7 cells with the axis on the left; dot: culture suspension with the axis on the right. (d) Detection of negative-strand RNA in the Huh7 cells by strand-specific RT-PCR and nested PCR. I: Huh7 cells infected with purified HCV virions 6 d post infection; II: Huh7 cells infected with culture suspension from the infection system 6 d post incubation; a: strand-specific RT-PCR, 375 bp; b: nested PCR, 297 bp; ck: natural Huh7 cells. GAPDH played as system control

and reduced to $(1.93 \pm 0.92) \times 10^4$ copies/ μ g of total RNA at 8 d (Fig. 5c). However, the copies of HCV RNA in the culture suspension increased with the elapse of time and peaked at 8 d post infection (Fig. 5c), suggesting that HCV virions were released into the culture suspension with the prolongation of cultivation. And we also confirmed that the culture suspension was infective since we detected both HCV negative-strand RNA (Fig. 5d) and proteins (E2 and NS5B) in the natural Huh7 cells infected with the culture suspension (Fig. 6a-b and d). Therefore, HCV virions produced from the Vero culture system could infect the natural Huh7 cells and replicate efficiently.

HCV protein expression in the infected Huh7 cells

Western blot analysis detected the expression of HCV protein in the infected Huh7 cells. Results shown in Fig. 6a

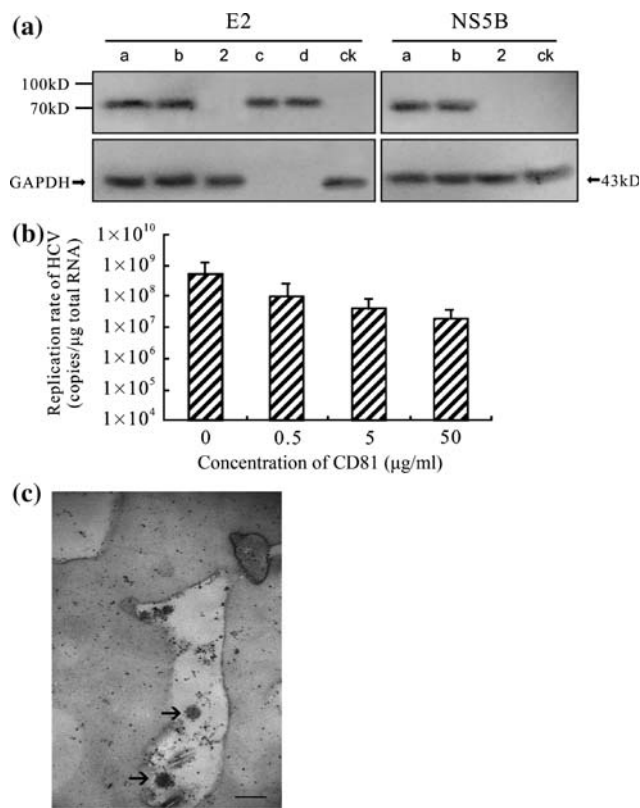


Fig. 6 HCV particles infectivity test and electron microscopic observation. **(a)** Western blot analysis of infected Huh7 cells for HCV proteins E2 and NS5B. a: Huh7 cells infected with purified HCV virions 6 d post infection; b: Huh7 cells infected with culture suspension from the infection system 6 d post incubation; c: culture suspension of “a” 6 d post infection; d: culture suspension of “b” 6 d post incubation. 2: Huh7 cells infected with HCV virus which was supplemented with anti-E2 monoclonal antibody overnight; ck: natural Huh7 cells. GAPDH played as system control. **(b)** Inhibition of HCV infection by anti-CD81 antibody. HCV RNA was analyzed by fluorescent quantitative PCR at 3 d post infection. The data of the column are the average of two detections. The error bars are at the top of the columns. **(c)** Electron microscopy observation of HCV particles in infected Huh7 cells. \uparrow : HCV particles, scale bar represents 100 nm

demonstrated that both HCV structure (E2 72 kD) and nonstructure protein (NS5B 68 kD) could be detected in the Huh7 cells at 6 d post infection (Fig. 6a-a). Furthermore, the E2 protein could be detected in the culture suspension of the infected Huh7 cells (Fig. 6a-c), which proved that mature HCV virions were released into the culture suspension.

HCV infection is inhibited by anti-E2 antibodies

Previous studies have suggested that the envelope E2 protein is necessary for HCV entry in the host cell. So we detected the infectivity of HCV virions blocked by the anti-E2 antibodies. Results showed that the positive- and

negative-strand RNA (Fig. 5a and b line 2) were not detected, nor the proteins (Fig. 6a-2) in the infected Huh7 cells. This demonstrated that E2 protein is essential for HCV infection and the HCV virions produced from the Vero culture system were intact and infective.

HCV infection is inhibited by anti-CD81 antibodies

Previous studies using pseudotype viruses that express HCV E1/E2 have also suggested that the interaction between HCV E2 and CD81 is crucial for viral entry [19]. To determine whether CD81 is required in this HCV infection system, Huh7 cells treated with anti-CD81 antibody were infected with HCV virions and analyzed 3 d post infection. Intracellular HCV RNA levels were reduced in a dose-dependent manner with a 25-fold reduction at 50 μ g/ml anti-CD81 antibody as compared with the control cells (Fig. 6b).

HCV virion production and electron microscopic observation

We successfully observed HCV particles in the infected Huh7 cell cultured for 4 d (Fig. 6c), the virions were located in the cytoplasm with a diameter of about 55 nm which is the same as in the transfected Vero cells.

From all the experiments that we have described, we argue that the HCV virions from the cultured Vero system have excellent infectivity to Huh7 cells.

Discussion

Although the cell line has recently been reported to be capable of producing HCV-like particles [16], it is still a question of whether the virus could be produced in the Vero cell—a normative host in the vaccine production or not. Furthermore, previous studies with a genotype 1b virus suggest mutations can promote RNA replication in cultured cells but reduce the ability of the virus to infect chimpanzees [20, 21]. Therefore, a noncancerous original cell model system with viral replication, assembly, and release is urgently needed.

Here, we reported the development of a new Vero cell culture system for HCV 1b production without adaptive mutations. The efficiency of HCV replication was well over 10^8 copies of positive RNA per milliliter. In addition, the infection experiment showed that HCV virions produced by the pHCV-WHU-1 were infectious. There are three distinctive features of our cell culture system.

- (1) Construction of full-length HCV cDNA genome clone (1b) without adaptive mutations. HCV particles releasing from Vero cell membrane was observed (Fig. 4b). It is progress compared with the previous model systems [22–24].
- (2) The production of large number of copies of HCV RNA based on the specific regulation of recombinant vaccine virus [25]. The high expression of the T7 polymerase offered by ν TF7-3 is the key to enhancing the copies of HCV RNA in our cell culture system.
- (3) Vero cell line was used as the host cell to produce HCV virions in the system. Vero cells have short proliferation cycle, and they are hypersensitive to ν TF7-3 as well, which renders it being one of the safest host cell lines used to produce vaccines such as poliomyelitis virus.

In our cell culture system, not only the replication of HCV genome was enhanced significantly, but also structural and nonstructural proteins of HCV were processed properly (Fig. 3b). In addition, we detected the presence of negative-strand RNA in the transfected Vero cells (Fig. 2c) when exposed to Act.D, indicating that the pHCV-WHU-1 transfected into the Vero cells has the ability to self-replication.

Furthermore, the present HCV virion (about 55 nm) was confirmed by EM and IEM analyses (Fig. 4). The strongest evidence is the visualization of particles by electron microscopy, and these particles were visualized only in the fraction with the density of 1.17 g/ml (the published density of free HCV virions). Therefore, the HCV virions and the ν TF7-3 particles could be separated into different fractions by the sucrose density gradient centrifugation. Thus, the purified HCV virions were obtained to utilize in the infection experiments. The aforementioned evidence shows that the HCV virions from the Vero cell culture system have the same physiological characteristics as that of wild type.

For the cell culture system transfected with full-length HCV genome, an important issue is whether the HCV particles released from cells are infectious or not. In the infection experiments, we detect not only the synthesis of positive RNA but also the negative RNA (Fig. 5a and b). Furthermore, HCV proteins were detected (Fig. 6a), which indicated that HCV virions from the Vero cell culture system were infectious, replicative-competent and could express proteins in a permissive cell line. In addition, we also observed HCV particles in the infected Huh7 cytoplasm (Fig. 6c). It has the same configuration as that of the virions in the transfected Vero cell, indicating that HCV virions produced from the Vero cell culture system have the ability to infect the host cell Huh7 and complete their life cycle to produce the next generation. Moreover, the

copies of HCV RNA in the culture suspension increased with the elapse of time, suggesting that the HCV virions have completed their life cycle and released into the suspension. The cultured suspension is infectious for natural Huh7 cells since we could detect both negative-strand HCV RNA (Fig. 5d) and proteins in the natural Huh7 cells (Fig. 6a–b and d). Results showed that we could detect neither the HCV RNA nor the proteins when HCV particles were neutralized by anti-E2 monoclonal antibody. This demonstrated that the HCV virions from the Vero culture system were intact and the E2 protein was essential for HCV entry its host cell to a certain extent.

HCV E2 has been shown to bind to the cellular surface protein CD81, which is an essential co-receptor for the entry of HCV glycoprotein-pseudotyped retroviruses [19]. Consistent with this statement, HCV infection of Huh7 cells was inhibited by an antibody to CD81, an extensively characterized putative HCV receptor (Fig. 6b). When it comes to the virus, correct packaging and release from the host are important process for the virus to proliferate successfully. The vaccine often targets to the interrelated virus proteins which is functional during virus infection. As the HCV virions produced from the Vero cell culture system have good infectivity, it will be helpful to vaccine production consequently.

Conclusion

In conclusion, this novel model system has wide practicability and applicability. This work presents an effective system to obtain purified HCV virions from the full-length cDNA clone pHCV-WHU-1. Huh7 cell infection experiments show that the HCV virions from the cultured Vero system present natural integrality and infectivity. Furthermore, it offers an opportunity to improve the vaccine research as well as the therapeutic targets for the treatment of Hepatitis C.

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