Available online at www.sciencedirect.com
SCIENCE DIRECT®
Antiviral Research 58 (2003) 199–208
Antiviral Research
www.elsevier.com/locate/antiviral

An in vitro system combined with an in-house quantitation assay for screening hepatitis C virus inhibitors

Tin-Yun Ho [a], Shih-Lu Wu [b], I-Lu Lai [c], Ken-Sheng Cheng [d], Shung-Te Kao [a], Chien-Yun Hsiang [c,*]

[a] *Institute of Chinese Medical Science, China Medical College, Taichung, Taiwan, ROC*
[b] *Department of Biochemistry, China Medical College, Taichung, Taiwan, ROC*
[c] *Department of Microbiology, China Medical College, 91 Hsueh-Shih Road, Taichung 404, Taiwan, ROC*
[d] *Division of Gastroenterology, Department of Internal Medicine, China Medical College Hospital, Taichung, Taiwan, ROC*

Received 24 September 2002; accepted 3 December 2002

Abstract

Hepatitis C virus (HCV) infection is a serious global health problem. Interferon-α (IFN-α) and ribavirin have demonstrated efficacy in the treatment of HCV infection; however, these therapies display many side effects. To screen the anti-HCV compounds from plants, we established an in vitro model for inoculation of HCV by centrifugation-facilitated method. The HCV RNA molecules were then quantitated by nested competitive reverse transcription-polymerase chain reaction (cRT-PCR) using fluorescein-labeled primers, and analyzed by ABI Prism™ 310. The positive and negative strands of HCV RNA were detectable in Vero cells on Day 7 post-infection, suggesting that the HCV RNA was present in the cell model system. The cell culture system was further used to screen the anti-HCV activities of 4 Chinese herbal formulas and 15 formula components. IFN-α showed an antiviral effect. The formulas exhibited no anti-HCV activities, while *Arnebia euchroma*, *Thlaspi arvense*, and *Poncirus trifoliata* displayed anti-HCV activities. Therefore, these results pointed out the possibility by using the cell culture system established in this study to screen the herb extracts for their anti-HCV activities.

Keywords: Hepatitis C virus; Nested competitive reverse transcription-polymerase chain reaction; Centrifugation-facilitated inoculation; Chinese medicinal herbs

1. Introduction

Hepatitis C virus (HCV), a member of *Flaviviridae*, is the major causative agent of post-transfusion-associated non-A, non-B hepatitis (Choo et al., 1989). Most HCV infections are persistent, leading in about 50% of all cases to chronic hepatitis, which can develop into chronic active hepatitis, liver cirrhosis, and hepatocellular carcinoma (Alter et al., 1992). Chronic HCV infection in some cases is associated with systemic diseases, such as autoimmune diseases, cryoglobulinemia, porphyria cutanea tarda, and membranous glomerulonephritis (Alter et al., 1992). The World Health Organization (WHO) estimated that 3% of the world's population, approximately 170 million people, has been infected with HCV (WHO, 1997). In Taiwan, the prevalence of HCV in the general population is about 2.5% (Sheu et al., 1993). As estimated 5000–6000 deaths occur annually in Taiwan as a result of hepatitis and hepatocellular carcinoma (Chu et al., 1999).

Interferon-α (IFN-α) monotherapy and combination therapy of ribavirin plus intron-A (Rebetron) are the only FDA-approved agents that have demonstrated efficacy in the treatment of HCV infection (Rosen and Gretch, 1999). The IFN-α treatment leads to a sustained clearance of HCV RNA in 15–20% of patients (Hoofnagle and Lau, 1996). However, IFN therapy is associated with many side effects (especially after prolonged therapy) and is effective in only a subset of patients (Martinot Peignoux et al., 1998). The combination therapy shows a 28–66% sustained response after 48 weeks of treatment (McHutchison et al., 1998); however, ribavirin frequently causes hemolytic anemia and is a known teratogen (Sherman, 1999). Thus, the development of new therapeutic agents against HCV is required.

Screening of the antiviral compounds required an efficient cell culture system for propagation of virus. HCV has been shown to replicate in a limited number of cultured

* Corresponding author. Tel.: +886-4-22053366x8503; fax: +886-4-22053764.
E-mail address: cyhsiang@mail.cmc.edu.tw (C.-Y. Hsiang).

0166-3542/03/$ – see front matter © 2003 Elsevier Science B.V. All rights reserved.
doi:10.1016/S0166-3542(03)00004-4

cell types, including primary human and chimpanzee liver cells (Ito et al., 1996; Lanford et al., 1994), hepatocarcinoma cell lines (Song et al., 2001; Tsuboi et al., 1996), human hematopoietic cell lines (Cribier et al., 1995), and human fibroblast cells (Zibert et al., 1995). However, thus far these systems suffer from a poor reproducibility and a low level of HCV replication that can be measured only with highly sensitive techniques. Centrifugation of the sample inoculum onto cell monolayers has been shown to increase the efficiency of in vitro isolation of a variety of virus, including cytomegalovirus, herpes simplex virus, influenza virus, enteroviruses and adenoviruses, by shortening virus detection times and enhancing recovery rates (Brumback and Wade, 1994; Klespies et al., 1996; Ziegler et al., 1995). It will, therefore, be benefit in establishing a cell culture system for HCV propagation.

In this study, we set up an in vitro model for infection of HCV and established a sensitive method to quantitate HCV RNA molecules in the cultures. The HCV-positive serum was inoculated to Vero cells by centrifugation-facilitated method. The HCV RNA molecules in the cultures were quantitated by nested competitive reverse transcription-polymerase chain reaction (cRT-PCR) using fluorescein-labeled primers and analyzed by ABI Prism™ 310 (Applied Biosystems). The in vitro model was further applied to screen the Chinese herbal formulas and herb components for their anti-HCV activities.

2. Materials and methods

2.1. Serum specimens

HCV RNA-positive sera were collected from the patients with historically proven hepatitis C. HCV status was determined by Amplicor HCV Monitor™ (Roche Diagnostic Systems). The sera were stored in aliquots at −80 °C until use.

2.2. Cell line and virus inoculation

African green monkey kidney cells (Vero cells) were grown in Dulbecco's minimum essential medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Virus inoculation was performed by centrifugation-facilitated method as described previously (Klespies et al., 1996). Briefly, Vero cells (5×10^6), grown in 25-T flasks for less than 2 days, were inoculated with 1 ml of diluted serum and incubated at room temperature with gentle shaking. After a 30-min incubation, the cells were centrifuged at 2000 × *g* at room temperature for 45 min. The inocula were then removed, the monolayers were rinsed once with DMEM, and 5 ml of DMEM supplemented with 10% FBS was added. After a 24-h incubation at 37 °C, the cells were harvested for the quantitation of HCV RNA by nested cRT-PCR.

2.3. Extraction of RNA

RNAs were extracted from serum or infected cells according to the acid guanidinium–phenol–chloroform (AGPC) method (Chomczynski and Sacchi, 1987). Briefly, 50 μl of serum, mixed with 450 μl of solution D (4 M guanidinium thiocyanate, 25 mM sodium citrate (pH 7.0), 0.5% sarkosyl, 0.1 M 2-mercaptoethanol), 50 μl of 3 M sodium acetate (pH 4.0), 0.5 ml of phenol (pH 4.0), and 0.1 ml of chloroform–isoamyl alcohol (49:1), was vortexed and centrifuged at 10,000 × *g* for 20 min at 4 °C. The RNA was then precipitated by isopropanol, dissolved in RNase-free water, and stored at −80 °C in small aliquots. For preparing the RNA from infected cells, the cells were washed once with ice-cold phosphate-buffered saline and lysed by 500 μl of solution D. The RNA was extracted as described earlier.

2.4. Quantitation of HCV RNA

2.4.1. Construction and in vitro transcription of competitor RNA

HCV RNA was reverse transcribed by SuperScript™ II (GibcoBRL) and then amplified for 35 cycles with F1 (5′-AACTACTGTCTTCACGCAGAA-3′; positions −289 to −269) and R1 (5′-GATGCACGGTCTACGAGACCTC-3′; positions −1 to −22) primers. The 289-bp product was ligated into the *Sma*I site of pBluescript® II KS(−) to create an HCV5F1. HCV5F1 was further digested with *Ppu*MI and *Nhe*I, which cleaved a 60-bp fragment inside the 289-bp product, and ligated to yield an internal truncated plasmid HCV5F1a. Competitor RNA was transcribed from HCV5F1a using T7 RNA polymerase and Riboprobe® In Vitro Transcription Systems (Promega) according to the manufacturer's protocol. To purify the competitor RNA from template DNA, the synthetic RNA was treated with 50 U of deoxyribonuclease I (Roche) at 37 °C for 10 min, followed by phenol–chloroform extraction and ethanol precipitation. The copy numbers of competitor RNA were defined according to conventional methods.

2.4.2. Nested cRT-PCR

cDNAs were synthesized by using R1 primer in the presence of HCV genomic RNA and competitor RNA. After a 1-h reverse transcription at 42 °C, the amplification reaction was carried out for 35 cycles in a 100-μl mixture containing 10 mM NaCl, 5 mM Tris–HCl (pH 8.0), 0.1% Triton® X-100, 1.5 mM $MgCl_2$, 2.5 U Taq DNA polymerase (Promega), and 10 pmol each of the F1 and R1 primers. The amplification profile was as follows: denaturation at 94 °C for 60 s, annealing at 55 °C for 60 s, and extension at 72 °C for 90 s. The 10-μl product was further re-amplified for 35 cycles with internal primers R2 (5′-CACTCTCGAGCACCCTATCAGGCAGT-3′; positions −29 to −54) and 6-carboxyfluorescein (FAM)-labeled F2 (5′-TTCACGCAGAAAGCGTCTAG-3′; positions −279 to

−260) to obtain a 251-bp wild-type fragment and a 191-bp competitor fragment.

2.4.3. Competition analysis

After PCR amplification, 10-μl aliquots of the reaction mixture were run on a 2.5% agarose gel at 50 V for 45 min and stained with ethidium bromide to visualize the wild-type and competitor fragments. For quantitation, 2-μl aliquots of mixture were mixed with 12 μl of deionized formamide and 1 μl of TAMRA-500, and analyzed by ABI Prism™ 310 (Applied Biosystems). The peak areas (Wa = wild-type area; Ca = competitor area) of both amplified products were calculated by GeneScan® Analysis program (Applied Biosystems). The Ca/Wa ratio was calculated from each sample and was plotted on the *y* axis against the copy number of the RNA competitor. The copy number of the wild-type template could be calculated from the curve expression for Ca/Wa = 1.

2.5. Detection of positive and negative strands of HCV RNA

To detect the positive stranded HCV RNA, 1 μg of RNA was reverse transcribed with the antisense outer primer (R1). The cDNA was then amplified by F1 and R1, and re-amplified by internal primers F2 and R2. To detect the negative stranded HCV RNA, 1 μg of RNA was reverse transcribed by using the sense outer primer (F1) as the primer. The cDNA was then amplified by nested RT-PCR as describe earlier. The β-actin RNA was reverse transcribed with the oligo$(dT)_{16}$ primer. The cDNA was then amplified by P1 (5′-TCCTGTGGCATCCACGAAACT-3′) and M1 (5′-GAAGCATTTGCGGTGGACGAT-3′). β-Actin was used as a housekeeping control. The products were analyzed by 2.5% agarose gels and visualized by ethidium bromide staining.

2.6. Preparation of herb extracts

Four Chinese herbal formulas and 15 formula components were extracted and analyzed for their anti-HCV activities. Su-ni-san was composed of *Paeonia lactiflora* Pall., *Bupleurum chinense* DC., *Glycyrrhiza uralensis* Fisch., and *Poncirus trifoliata* Raf. Chia-wei-su-ni-san was made up of eight herbs, including Su-ni-san supplemented with *Polygonum cuspidatum* SI., *Thlaspi arvense* L., *Arnebia euchroma* Johnst., and *Astragalus membranacens* Bge., and was mixed in a weight ratio of 3:3:3:3:3:3:2:3. Hsia-yao-san was comprised of *B. chinense* DC., *Angelica sinensis* Diels, *P. lactiflora* Pall., *Atractylodes ovata* DC., *Poria cocos* Wolf, *Mentha arvensis* L., *Zingiber officinale* Rosc., and *G. uralensis* Fisch. Chia-wei-hsiao-yao-san was made up of 12 herbs, including Hsia-yao-san supplemented with *P. cuspidatum* SI., *T. arvense* L., *Salvia miltiorrhiza* Bge., and *Imperata cylindrica* L., and was mixed in a weight ratio of 4:4:4:4:4:2:4:2:4:4:3:2. The plant materials were gifts from Sun Ten Pharmaceutical Corporation (Taiwan). Plant samples were ground with homogenizer to a fine powder. The methanolic extract was prepared by mixing 3 g of each herb powder with 10 ml of 100% methanol and shaking at 4 °C overnight. All extracts were centrifuged at 10,000 × *g* for 5 min, and the supernatants were then evaporated under vacuum to dryness and resuspended in dimethyl sulfoxide (DMSO) to a final concentration of 1 mg/ml. The extracts were stored at −20 °C in small aliquots.

2.7. Cytotoxicity assay

Vero cells were cultivated in 96-well culture plates. After a 24-h incubation at 37 °C in a humidified CO_2 atmosphere (5% CO_2), serial twofold dilutions of the extracts were added to confluent cell monolayers and incubated for another 24 h. The cytotoxicity was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay. Briefly, one-tenth volume of 5 mg/ml MTT was added to the culture medium. After a 4-h incubation at 37 °C, equal cell culture volume of 0.04 N HCl in isopropanol was added to dissolve the MTT formazan, and measured the absorbance at 570 nm using a microplate reader. The 50% toxicity concentration (TC_{50}) was determined as the quantity of drug required to reduce cell viability at 50%.

2.8. Antiviral assay

The virus was inoculated as described earlier. After a 24-h incubation at 37 °C, the cells were washed once with DMEM, one-eighth TC_{50} of herb extract was added to cells, and the cells were incubated at 37 °C for another 72 h. For IFN-α treatment, the cells were washed once with DMEM, treated with various amounts of IFN-α, and then incubated at 37 °C for 3 days. HCV RNAs were extracted from cells and quantitated by nested cRT-PCR. IFN-α was purchased from Sigma Chemical Co. (St. Louis, MO).

2.9. Statistical analysis

The antiviral activities of herb extracts were calculated by the Ca/Wa ratio. The significant difference in the antiviral ability was evaluated by Student's *t*-test.

3. Results

3.1. Quantitation of HCV RNA molecules by nested cRT-PCR

We established a nested cRT-PCR technique to quantitate HCV RNA molecules in this study. The competitor was designed as an internal truncated fragment to ensure the co-amplification of wild-type RNA using the same set of primers. Moreover, it contained a G + C content

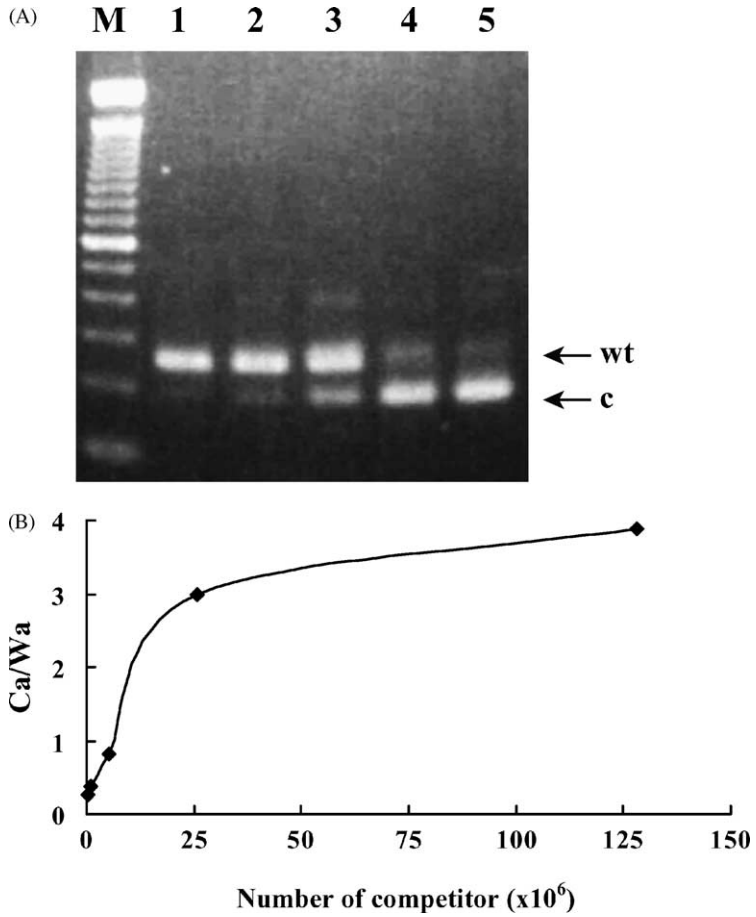


Fig. 1. Quantitation of HCV genome molecules in serum samples by nested cRT-PCR. (A) Agarose gel analysis. HCV RNA, extracted from 50 μl serum, was co-amplified with 2.05×10^5 (lane 1). 1.02×10^6 (lane 2), 5.12×10^6 (lane 3), 2.56×10^7 (lane 4), or 1.28×10^8 (lane 5) copies of competitor RNA by nested cRT-PCR with fluorescein-labeled primers. The products were analyzed by 2.5% agarose gels and visualized by ethidium bromide staining. The 251-bp wild-type fragment (wt) and 191-bp competitor fragment (c) are indicated by arrows. Lane M represents the 100-bp DNA ladder. (B) Quantitative analysis. The amplified products were analyzed by ABI Prism™ 310. The peak areas of wild-type product (Wa) and competitor product (Ca) were quantitated by GeneScan analysis software. The Ca/Wa value was plotted against the competitor copy number in the respective graphs.

similar to that in amplicates from HCV RNA templates to avoid the amplification bias. HCV RNA was extracted from HCV-positive serum by AGPC method instead of commercial kit (QIAamp® Viral RNA Mini Kit; Qiagen) because the RNA carrier in the precipitation solution interfered with the following reaction (data not shown). HCV RNA, extracted from 50 μl of HCV-positive serum, was co-amplified with a serial dilution of competitor by nested cRT-PCR with fluorescein-labeled primers. Fig. 1A shows that the amount of wild-type product was gradually decreased as the copy number of competitor increased. The amount of wild-type product (Wa) and competitor product (Ca) was further quantitated by ABI Prism™ 310 using GeneScan analysis software. The Ca/Wa ratio was calculated and was plotted on the *y* axis against the copy number of competitor RNA (Fig. 1B). The copy number of the wild-type template could be calculated from the curve expression for Ca/Wa = 1. The data showed that HCV RNA concentration was 6.5×10^6 molecules, which is equal to 1.3×10^8 copies/ml serum. Similar HCV titer (1×10^8 copies/ml) was measured by branched DNA assay (bDNA) (Quantiplex™ HCV RNA 2.0; Bayer Diagnostic) (data not shown).

To further assess the precision and sensitivity of nested cRT-PCR, a defined amount (100 molecules) of HCV RNA was co-amplified with a serial dilution of competitor by nested cRT-PCR and quantitated as aforementioned. The HCV RNA concentration was calculated to be 100 molecules as expected when the Ca/Wa ratio equals 1 (Fig. 2), indicating the accuracy of nested cRT-PCR in quantifying the HCV RNA molecules. Moreover, the data also showed that the detection and quantitation ranges of HCV RNA by nested cRT-PCR were from 100 to 1.3×10^8 copies/ml.

3.2. Optimization of inoculation condition for HCV infection in cultures

To screen the anti-HCV effect of Chinese herbal formulas and herbs, we optimized the inoculation conditions

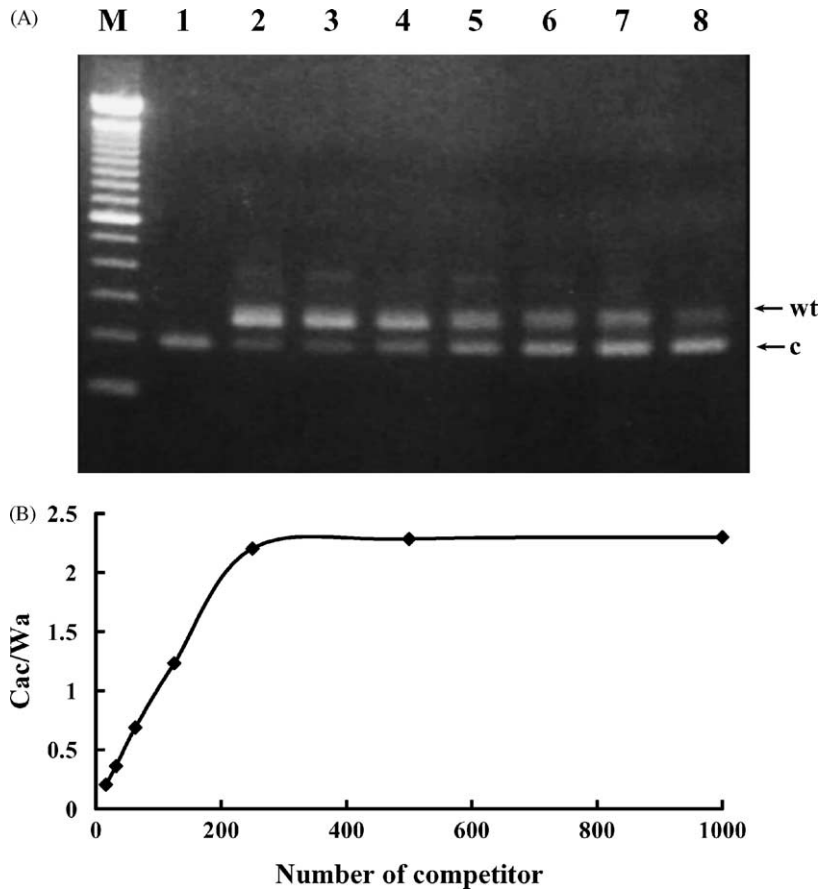
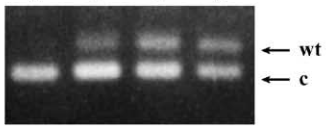


Fig. 2. Quantitation of HCV genome molecules by nested cRT-PCR. (A) Agarose gel analysis. HCV RNA (100 molecules) was co-amplified with 16 (lane 2), 32 (lane 3), 63 (lane 4), 125 (lane 5), 250 (lane 6), 500 (lane 7), or 1000 (lane 8) copies of competitor RNA by nested cRT-PCR with fluorescein-labeled primers. The products were analyzed by 2.5% agarose gels and visualized by ethidium bromide staining. The 251-bp wild-type fragment (wt) and 191-bp competitor fragment (c) are indicated by arrows. Lane M represents the 100-bp DNA ladder. Lane 1 represents the amplification of 200 copies of competitor RNA. (B) Quantitative analysis. The amplified products were analyzed by ABI Prism^{TM} 310. The peak areas of wild-type product (Wa) and competitor product (Ca) were quantitated by GeneScan analysis software. The Ca/Wa value was plotted against the competitor copy number in the respective graphs.

for HCV infection in cultures. The virus inoculation was carried out by centrifugation-facilitated method and the HCV RNA concentration was quantitated by the Ca/Wa ratio. When the Ca/Wa ratio equaled 1, the copy number of HCV genome was the same as that of competitor RNA. When the Ca/Wa ratio was more than 1, the copy number of HCV RNA was less than that of competitor RNA. In other words, the more the Ca/Wa ratio was, the less the copy number of HCV genome was. We first tested the optimal inoculation time for HCV infection. The Vero cells were inoculated with 1 ml of diluted serum, incubated at room temperature for 30 min to allow virus adsorption, and subsequently incubated at 37 °C for 45 or 90 min to allow penetration. After a 45-min centrifugation, the infected cells were cultivated for 24 h and the HCV RNA in cells was quantitated by nested cRT-PCR (Fig. 3A). The Ca/Wa ratio of HCV infection at room temperature (8.25 ± 0.67) was significantly higher than the one at room temperature and subsequently at 37 °C, indicated that a 37 °C-incubation enhanced the HCV infection in culture. Moreover, the Ca/Wa ratio of HCV infection at 37 °C for 45 min (3.60 ± 0.09) was more than the one for 90 min (2.91 ± 0.32), showed that the HCV RNA concentration in culture was higher in a 90-min incubation at 37 °C than a 45-min incubation at 37 °C.

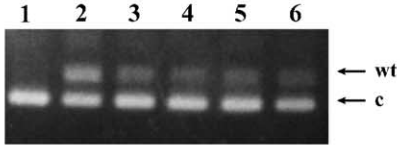
For detecting the effect of dose (multiplicity of infection (m.o.i.)) on HCV infection, Vero cells (5×10^6) were inoculated with 1 ml of diluted serum containing 2.6×10^7, 1.3×10^7, 6.5×10^6, 3.3×10^6, or 1.6×10^6 HCV RNA molecules, which equaled to 5.2, 2.6, 1.3, 0.7, or 0.3 m.o.i., respectively, and the HCV RNA was quantitated by nested cRT-PCR (Fig. 3B). The data showed that HCV RNA was detectable at 0.3 m.o.i. The copy number of HCV RNA infected at 0.3 m.o.i. was significant lower than the one at 5.2 m.o.i. However, because the difficulty in acquiring the HCV-positive serum, the m.o.i. used for HCV infection was 1.3. The Vero cells were further inoculated with various volumes of diluted serum at an equal m.o.i., incubated

(A)



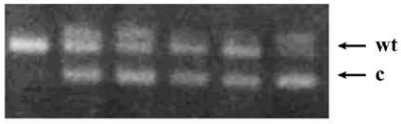
Lane	Ca/Wa
2	8.25±0.67
3	3.6±0.09
4	2.91±0.32

(B)



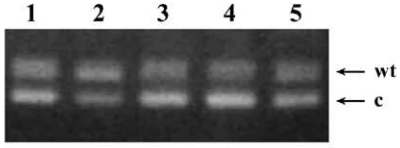
Lane	Ca/Wa
2	1.73±0.28
3	4.45±0.40
4	6.62±1.01
5	7.79±0.64
6	9.98±0.87

(C)



Lane	Ca/Wa
1	0.06±0.02
2	1.04±0.07
3	1.82±0.15
4	1.32±0.07
5	1.18±0.16
6	3.09±0.42

(D)



Lane	Ca/Wa
1	2.41
2	2.35
3	2.98
4	2.65
5	2.29

Fig. 3. Optimal conditions for HCV infection in culture. (A) Inoculation time. Vero cells were inoculated with 1 ml of diluted serum and incubated at room temperature for 30 min (lane 2), or incubated at room temperature for 30 min followed by incubating at 37 °C for 45 min (lane 3) or 90 min (lane 4). RNAs were extracted from infected cells or mock cells (lane 1), and co-amplified with 200 copies of competitor RNA. (B) Multiplicity of infection (m.o.i.). Vero cells (5×10^6) were incubated with 1 ml of diluted serum containing 2.6×10^7 (lane 2), 1.3×10^7 (lane 3), 6.5×10^6 (lane 4), 3.3×10^6 (lane 5), or 1.6×10^6 (lane 6) of HCV RNA molecules, incubated at room temperature for 30 min, and centrifuged at $2000 \times g$ for 45 min. RNAs were extracted from infected cells or mock cells (lane 1), and co-amplified with 200 copies of competitor RNA. (C) Inoculation volume. Vero cells were inoculated with 1 (lane 1), 2 (lane 2), 3 (lane 3), 4 (lane 4), 5 (lane 5), or 6 ml (lane 6) of diluted serum at an equal m.o.i. and incubated at room temperature for 30 min. RNAs were extracted and co-amplified with 200 copies of competitor RNA. (D) Homogeneity test. Vero cells (5×10^6) were infected with HCV at 1.3 m.o.i. in five independent tests. RNAs were extracted from infected cells (lanes 1–5) and co-amplified with 400 copies of competitor RNA. The products were analyzed by 2.5% agarose gels and visualized by ethidium bromide staining. The 251-bp wild-type fragment (wt) and 191-bp competitor fragment (c) are indicated by arrows. The Ca/Wa ratio of each lane is presented at the right.

at room temperature for 30 min, and subsequently at 37 °C for 90 min. Fig. 3C shows that the copy number of HCV genome in cells decreased as the incubation volume increased.

To apply the cell culture system in screening the anti-HCV herbs, it is important to ensure the reproducibility and homogeneity of lots of tests. We therefore carried out the homogeneity test for five independent experiments. Vero cells were inoculated with 1 ml of diluted serum at 1.3 m.o.i., incubated at room temperature for 30 min, and subsequently incubated at 37 °C for 90 min. After a 45-min centrifugation, the infected cells were cultivated for 24 h and the HCV RNA was quantitated by nested cRT-PCR. Fig. 3D shows the results from five independent experiments. The Ca/Wa ratios of independent experiments showed no significant variance, suggested that the cell culture system for HCV infection established in this study could be used to screen the anti-HCV herbs.

3.3. Detection of positive and negative strands of HCV RNA in the cultures

To test whether the propagation of HCV has occurred, the time course experiment was carried out. Fig. 4 shows that positive strand of HCV RNA was detectable in cells on Day 0, 3, and 7 post-infection. Because the product was analyzed during the plateau phase of nested RT-PCR, there was no increase in the amount of positive stranded HCV RNA in the time course experiment. The negative strand of HCV RNA was detected until Day 3 post-infection. To avoid the false priming or self-priming of minus strand RNA, we synthesized the cDNA in the absence of primer F1 and amplified the cDNA by nested PCR. No product was observed in gels (data not shown), indicating the accuracy of strand specificity during nested RT-PCR. The appearance of negative stranded RNA is the indicator of viral RNA replication, it therefore suggested that HCV is probably replicating in this cell model system.

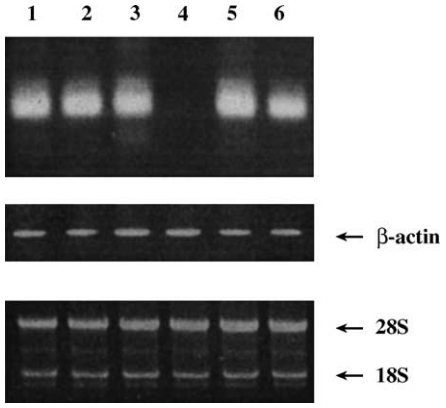


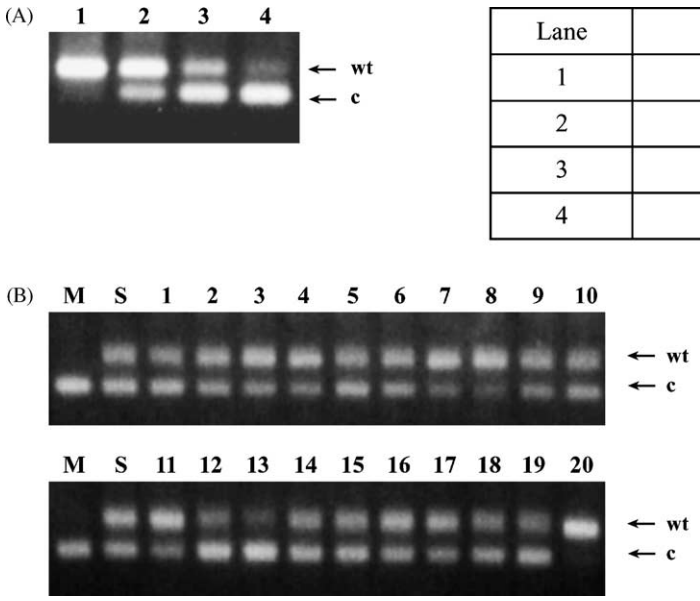
Fig. 4. Detection of positive and negative strands of HCV RNA in culture. Vero cells were infected with HCV, washed once with DMEM, and then incubated at 37 °C for 0 day (lanes 1 and 4), 3 days (lanes 2 and 5), or 7 days (lanes 3 and 6). Positive stranded HCV RNA (lanes 1–3) and negative stranded HCV RNA (lanes 4–6) were reverse transcribed by using R1 and F1 as the primer, respectively. The cDNAs were then amplified as described in Section 2. The products were analyzed by 2.5% agarose gels and visualized by ethidium bromide staining. The internal control (β-actin) and ribosomal control (28S and 18S) are indicated by arrows.

Table 1
Toxicity concentration of Chinese herbal formulas and herb extracts in Vero cells

	TC_{50} (μg/ml)
Hsiao-yao-san	53
Chia-wei-hsiao-yao-san	98
Su-ni-san	32
Chia-wei-su-ni-san	34
Mentha arvensis	21
Glycyrrhiza uralensis	16
Paeonia lactiflora	265
Poria cocos	10
Salvia miltiorrhiza	9
Polygonum cuspidatum	29
Poncirus trifoliata	93
Arnebia euchroma	4
Bupleurum chinense	12
Angelica sinensis	39
Atractylodes ovata	22
Zingiber officinale	26
Imperata cylindrica	75
Thlaspi arvense	48
Astragalus membranacens	61

3.4. Screening the anti-HCV effects of Chinese herbal formulas and herbs

The cytotoxicity of herb extracts as shown on Table 1 was determined using Vero cells to obtain the TC_{50}. The anti-HCV effect of each herb was assayed using the concentration at one eighth of TC_{50}. The HCV-infected cells were incubated with herb extracts and IFN-α for 72 h because the antiviral ability of IFN-α showed more effective on Day 3 in other study (Blight et al., 2000). The HCV RNA concentration was then determined by nested cRT-PCR. IFN-α reduced the concentration of HCV RNA in a dose-dependent manner (Fig. 5A). The formulas (Hsia-yao-san, Chia-wei-hsiao-yao-san, Su-ni-san, Chia-wei-su-ni-san) exhibited no anti-HCV activities, while the *A. euchroma* and *P. trifoliata* from Chia-wei-su-ni-san, and *T. arvense* from Chia-wei-hsiao-yao-san and Chia-wei-su-ni-san displayed significant anti-HCV activities than solvent. *Astragalus membranacens* from Chia-wei-su-ni-san enhanced the HCV infection in Vero cells (Fig. 5B and Table 2). Moreover, ribavirin exhibited no anti-HCV activity, which is consistent with other studies (Lee et al., 1998; Zoulim et al., 1998). Therefore, these results pointed out the possibility by using the cell culture system to screen the herb extracts for their anti-HCV activities.



Lane	Ca/Wa
1	0.22
2	0.85
3	1.81
4	3.69

Fig. 5. Effect of IFN-α and herb extracts on HCV infection in culture. (A) IFN-α treatment. Vero cells were infected with HCV and treated with 0 (lane 1), 10 (lane 2), 100 (lane 3), or 1000 IU/ml (lane 4) IFN-α. After inoculating at 37 °C for 3 days, the RNA was extracted and co-amplified with 400 copies of competitor RNA. The Ca/Wa ratio of each lane is presented at the right. (B) Herb extract treatments. Vero cells were infected with HCV and treated with solvent (DMSO) (lane S), ribavirin (lane 1), Hsiao-yao-san (lane 2), Chia-wei-hsiao-yao-san (lane 3), Su-ni-san (lane 4), Chia-wei-su-ni-san (lane 5), *Mentha arvensis* (lane 6), *Glycyrrhiza uralensis* (lane 7), *Paeonia lactiflora* (lane 8), *Poria cocos* (lane 9), *Salvia miltiorrhiza* (lane 10), *Polygonum cuspidatum* (lane 11), *Poncirus trifoliata* (lane 12), *Arnebia euchroma* (lane 13), *Bupleurum chinense* (lane 14), *Angelica sinensis* (lane 15), *Atractylodes ovata* (lane 16), *Zingiber officinale* (lane 17), *Imperata cylindrica* (lane 18), *Thlaspi arvense* (lane 19), or *Astragalus membranacens* (lane 20) for another 72 h. RNAs were extracted from mock cells (lane M) and treated cells, and co-amplified with 200 copies of competitor RNA. The 251-bp wild-type fragment (wt) and 191-bp competitor fragment (c) are indicated by arrows.

Table 2
Effect of formulas and herb extracts on HCV infection in culture

	Ca/Wa[a]
Solvent control	1.30 ± 0.16
Ribavirin	1.61 ± 0.22
Hsiao-yao-san	1.21 ± 0.03
Chia-wei-hsiao-yao-san	0.94 ± 0.18
Su-ni-san	1.08 ± 0.07
Chia-wei-su-ni-san	1.42 ± 0.09
Mentha arvensis	1.35 ± 0.17
Glycyrrhiza uralensis	0.91 ± 0.08
Paeonia lactiflora	0.62 ± 0.27
Poria cocos	1.09 ± 0.08
Salvia miltiorrhiza	1.37 ± 0.08
Polygonum cuspidatum	1.21 ± 0.22
Poncirus trifoliata	2.94 ± 0.62
Arnebia euchroma	5.28 ± 0.72[b]
Bupleurum chinense	1.83 ± 0.20
Angelica sinensis	1.73 ± 0.27
Atractylodes ovata	1.18 ± 0.10
Zingiber officinale	1.06 ± 0.03
Imperata cylindrica	1.69 ± 0.01
Thlaspi arvense	2.61 ± 0.11[b]
Astragalus membranacens	0.10 ± 0.04[b]

[a] Peak areas of competitor (Ca)/peak areas of wild-type (Wa); values are mean ± standard error of triplicate assays.
[b] $P < 0.05$ vs. solvent control by Student's *t*-test.

4. Discussion

This study described the establishment of an in vitro model for HCV inoculation and a nested cRT-PCR technique to quantitate HCV RNA molecules in the cultures. The HCV RNA has been quantitated by different techniques, either by commercial available tests or by in-house assays. The bDNA assay (Kolberg et al., 1994), based on solid-phase RNA hybridization and bDNA signal amplification, is developed as a simple assay kit for clinical use (Quantiplex™ HCV RNA 2.0; Bayer Diagnostic); however, it is less sensitive (3.5×10^5 copies/ml) (Shiratori et al., 1997). The Amplicor HCV Monitor™ (Roche Diagnostic Systems) (Miskovsky et al., 1996) and nucleic acid sequence-based amplification (NASBA-QT) (Damen et al., 1999) are based on HCV RNA amplification, and the quantitative detection limit is found to be 10^2–10^3 and 10^4 copies/ml, respectively (Damen et al., 1999). Recently, real-time detection-PCRs based on the TaqMan Chemistry System and LightCycler techniques are developed. It is based on the simultaneous amplification-detection of the target and quantitated the HCV RNA during the logarithmic phase of the reaction (Kishimoto et al., 2001; Nozaki and Kato, 2002). It is sensitive (25–300 copies/ml) (Martell et al., 1999; Nozaki and Kato, 2002); however, it needs an expensive detector to monitor the fluorescence intensity.

The nested cRT-PCR consists of carrying out reverse transcription and amplification in the presence of a known number of competitor template molecules that recognize the same primer (Haberhausen et al., 1998; Manzin et al., 1994). Its sensitivity was found to be 100 copies/ml, and the detectable ranges were from 100 to 1.3×10^8 copies/ml in this study. The copy number of HCV genome was determined by comparing the Ca/Wa ratio. Therefore, it is suitable to determine the HCV RNA concentration from a lot of samples by nested cRT-PCR.

Several studies described the establishment of cell lines for HCV replication (Blight et al., 2000; Ikeda et al., 1998; Seipp et al., 1997). Attempts have been made to use human cells of hepatocytic and lymphocytic origins, but low and variable levels of replication, and virus-induced cytotoxicity pose important problems (Leyssen et al., 2000). In recent years, centrifugation of the sample inoculum onto cell monolayers has acquired growing popularity in diagnostic virology because it has been shown to increase the efficiency of in vitro isolation of a variety of virus by shortening virus detection times and enhancing recovery rates (Morrica et al., 1999). We therefore set up an in vitro model for HCV infection by centrifugation-facilitated method in this study. The Vero and HepG2 cells have already been described as susceptible to HCV (Tagawa et al., 1995; Valli et al., 1997). We chose Vero cells instead of HepG2 cells for HCV infection because the low HCV concentration was found in HepG2 cells (data not shown). Vero cells were inoculated with diluted serum at 1.3 m.o.i., incubated at room temperature for 30 min, and subsequently incubated at 37 °C for 90 min. After a 45-min centrifugation at 2000 × *g*, the infected cells were cultivated for 24 h. The positive and negative strands of HCV RNA could be detectable on Day 7 post-infection, suggesting that HCV RNA is replicating in the cell model system.

The in vitro model for HCV infection and the quantitation technique established in this study were further applied in screening the herb extracts for their anti-HCV activities. Ribavirin was included as the control. Several studies indicated that ribavirin exhibited no anti-HCV effect (Lee et al., 1998; Zoulim et al., 1998). However, others showed that ribavirin exerted a direct effect on HCV replication, which was mediated by HCV RNA polymerase (Crotty et al., 2000; Maag et al., 2001). The Chinese herbal formulas—Hsiao-yao-san and Su-ni-san—are commonly used in the treatment of various chronic liver diseases in Taiwan. Glycyrrhizin, the major component of *G. uralensis*, has been clinically used for the treatment of chronic hepatitis B in Japan for years, because it modified the expression of hepatitis B virus-related antigens on the hepatocytes and suppressed the sialylation of hepatitis B virus surface antigen (Sato et al., 1996). *Salvia miltiorrhiza* has been demonstrated to exhibit the antioxidant capacity, inhibit the cell growth, and induce the apoptosis in human hepatoma HepG2 cells (Liu et al., 2000). Saponins, isolated from *B. chinense*, showed the hepatoprotective effect on liver injury induced by D-galactodamine and lipopolysaccharide in mice (Matsuda et al., 1997; Yoshikawa et al., 1997). The methanolic extracts of *A. euchroma* and *T. arvense* displayed significant anti-HCV activities ($P < 0.05$) after a 24-h treatment. The major components responsible for antiviral effect and the mechanism of antiviral activity of these herbs remained to be elucidated.

In conclusion, we set up an in vitro model for inoculation of HCV and established a sensitive method to quantitate HCV RNA molecules in the cultures. The HCV-positive serum was inoculated to Vero cells by centrifugation-facilitated method. The HCV molecules in the cultures were quantitated by nested cRT-PCR combined with ABI Prism™ 310. The in vitro model was further applied to screen the Chinese herbal formulas and herb components for their anti-HCV activities.

Acknowledgements

We thank Dr. S.R. Wang for technical support of bDNA assay. This work was supported by grants from National Science Council, China Medical College, and Committee on Chinese Medicine and Pharmacology at Department of Health, Taiwan.

References

Alter, M.J., Margolis, H.S., Krawczynski, K., Judson, F.N., Mares, A., Alexander, W.J., Hu, P.Y., Miller, J.K., Gerber, M.A., Sampliner, R.E., 1992. The natural history of community acquired hepatitis C in the United States. New Engl. J. Med. 327, 1899–1905.

Blight, K.J., Kolykhalov, A.A., Rice, C.M., 2000. Efficient initiation of HCV RNA replication in cell culture. Science 290, 196–198.

Brumback, B.G., Wade, C.D., 1994. Simultaneous culture for adenovirus, cytomegalovirus, and herpes simplex virus in same shell vial by using three-color fluorescence. J. Clin. Microbiol. 32, 2289–2290.

Chomczynski, P., Sacchi, N., 1987. Single-step method for RNA isolation by acid guanidinium thiocyanate–phenol–chloroform extraction. Anal. Biochem. 162, 156–159.

Choo, Q.L., Kuo, G., Weiner, A.J., Overby, L.R., Bradley, D.W., Houghton, M., 1989. Isolation of a cDNA clone derived from a blood-born non-A, non-B viral hepatitis genome. Science 244, 359–362.

Chu, C.M., Lin, S.M., Hsieh, S.Y., Yeh, C.T., Lin, D.Y., Sheen, I.S., Liaw, Y.F., 1999. Etiology of sporadic acute viral hepatitis in Taiwan: the role of hepatitis C virus, hepatitis E virus and GB virus-C/hepatitis G virus in an endemic area of hepatitis A and B. J. Med. Virol. 58, 154–159.

Cribier, B., Schmitt, C., Bingen, A., Kirn, A., Keller, F., 1995. In vitro infection of peripheral blood mononuclear cells by hepatitis C virus. J. Gen. Virol. 76, 2485–2491.

Crotty, S., Maag, D., Arnold, J.J., Zhong, W., Lau, J.Y., Hong, Z., Andino, R., Cameron, C.E., 2000. The broad-spectrum antiviral ribonucleoside ribavirin is an RNA virus mutagen. Nat. Med. 6, 1375–1379.

Damen, M., Sillekens, P., Cuypers, H.T.M., Frantzen, I., Melsert, R., 1999. Characterization of the quantitative HCV NASBA assay. J. Virol. Methods 82, 45–54.

Haberhausen, G., Pinsl, J., Kuhn, C.C., Markert-Hahn, C., 1998. Comparative study of different standardization concepts in quantitative competitive reverse transcription-PCR assays. J. Clin. Microbiol. 36, 628–633.

Hoofnagle, J., Lau, D., 1996. Chronic viral hepatitis-benefits of current therapies. New Engl. J. Med. 44, 1470–1471.

Ikeda, M., Sugiyama, K., Mizutani, T., Tanaka, T., Tanaka, K., Sekihara, H., Shimotohno, K., Kato, N., 1998. Human hepatocyte clonal cell lines that support persistent replication of hepatitis C virus. Virus Res. 56, 157–167.

Ito, T., Mukaigawa, J., Zuo, J., Hirabayashi, Y., Mitamura, K., Yasui, K., 1996. Cultivation of hepatitis C virus in primary hepatocyte culture from patients with chronic hepatitis C results in release of high titer infectious virus. J. Gen. Virol. 77, 1043–1054.

Kishimoto, H., Yoshioka, K., Yano, M., Ukai, K., Ito, H., Watanabe, K., Kawamata, O., Kakumu, S., 2001. Real-time detection system for quantitation of hepatitis C virus RNA: a comparison with the other three methods. Hepatol. Res. 19, 12–21.

Klespies, S.L., Cebula, D.E., Kelly, C.L., Galehouse, D., Maurer, C.C., 1996. Detection of enteroviruses from clinical specimens by spin amplification shell vial culture and monoclonal antibody assay. J. Clin. Microbiol. 34, 1465–1467.

Kolberg, J.A., Sanchez-Pescador, R., Detmer, J., Collins, M., Sheridan, P., Neuwald, P., Wilber, J., 1994. Branched DNA quantitation of hepatitis C viral RNA in patients sera. In: Group Francais d'Etudes Moleculaires des hepatites virales (GEMHEP) (Ed.), Hepatitis C Virus: New Diagnostic Tool. Jone Libbey Eurotext, Paris, pp. 57–70.

Lanford, R.E., Sureau, C., Jacob, J.R., White, R., Fuerst, T.R., 1994. Demonstration of in vitro infection of chimpanzee hepatocytes with hepatitis C virus using strand-specific RT-PCR. Virology 202, 607–614.

Lee, J.H., von Wagner, M., Roth, W.K., Teuber, G., Sarrazin, C., Zeuzem, S., 1998. Effect of ribavirin on virus load and quasispecies distribution in patients infected with hepatitis C virus. J. Hepatol. 29, 29–35.

Leyssen, P., de clercq, E., Neyts, J., 2000. Perspectives for the treatment of infection with Flaviviridae. Clin. Microbiol. Rev. 13, 67–82.

Liu, J., Shen, H.M., Ong, C.N., 2000. *Salvia miltiorrhiza* inhibits cell growth and induces apoptosis in human hepatoma HepG2 cells. Cancer Lett. 153, 85–93.

Maag, D., Castro, C., Hong, Z., Cameron, C.E., 2001. Hepatitis C virus RNA-dependent RNA polymerase (NS5B) as a mediator of the antiviral activity of ribavirin. J. Biol. Chem. 276, 46094–46098.

Manzin, A., Bagnarelli, P., Menzo, S., Giostra, F., Brugia, M., Francesconi, R., Bianchi, F.B., Clementi, M., 1994. Quantitation of hepatitis C virus genome molecules in plasma samples. J. Clin. Microbiol. 32, 1939–1944.

Martell, M., Gomez, J., Esteban, J.I., Sauleda, S., Quer, J., Cabot, B., Esteban, R., Guardia, J., 1999. High-throughput real-time reverse transcription-PCR quantitation of hepatitis C virus RNA. J. Clin. Microbiol. 37, 327–332.

Martinot Peignoux, M., Boyer, N., Pouteau, M., Castelnau, C., Giuily, N., Duchatelle, V., Auperin, A., Degott, C., Benhamou, J.P., Erlinger, S., Marcellin, P., 1998. Predictors of sustained response to alpha interferon therapy in chronic hepatitis C. J. Hepatol. 29, 214–223.

Matsuda, H., Murakami, T., Ninomiya, K., Inadzuki, M., Yoshikawa, M., 1997. New hepatoprotective saponins, bupleurosides III, VI, IX, and XIII, from Chinese Bupleuri Radix: structure-requirements for the cytoprotective activity in primary cultured rat hepatocytes. Bioorg. Med. Chem. Lett. 7, 2193–2198.

McHutchison, J.G., Gordon, S.C., Schiff, E.R., Shiffman, M.L., Lee, W.M., Rustgi, V.K., Goodman, Z.D., Ling, M.H., Cort, S., Albrecht, J.K., 1998. Interferon alpha-2b alone or in combination with ribavirin as initial treatment for chronic hepatitis C. New Engl. J. Med. 339, 1485–1492.

Miskovsky, E., Carrella, A., Gutekunst, K., Sun, C., Quinn, T., Thomas, D., 1996. Clinical characterization of a competitive PCR assay for quantitative testing of hepatitis C virus. J. Clin. Microbiol. 34, 1975–1979.

Morrica, A., Giorgi, M., Maggi, F., Fornai, C., Vatteroni, M.L., Marchi, S., Richiuti, A., Antonelli, G., Pistello, M., Bendinelli, M., 1999. Susceptibility of human and non-human cell lines to HCV infection as determined by the centrifugation-facilitated method. J. Virol. Methods 77, 207–215.

Nozaki, A., Kato, N., 2002. Quantitative method of intracellular hepatitis C virus RNA using LightCycler PCR. Acta Med. Okayama 56, 107–110.

Rosen, H.R., Gretch, D.R., 1999. Hepatitis C virus: current understanding and prospects for future therapies. Mol. Med. Today 5, 393–399.

Sato, H., Goto, W., Yamamura, J., Kurokawa, M., Kageyama, S., Takahara, T., Watanabe, A., Shiraki, K., 1996. Therapeutic basis of glycyrrhizin on chronic hepatitis B. Antiviral Res. 30, 171–177.

Seipp, S., Mueller, H.M., Pfaff, E., Stremmel, W., Theilmann, L., Goeser, T., 1997. Establishment of persistent hepatitis C virus infection and replication in vitro. J. Gen. Virol. 78, 2467–2476.

Sherman, A., 1999. HCV on the threshold. Infect. Med. 16, 92–94.

Sheu, J.C., Wang, J.T., Wang, T.H., Wang, C.Y., Yang, P.M., Huang, G.T., Shih, L.N., Lee, H.S., Chen, D.S., 1993. Prevalence of hepatitis C viral infection in a community in Taiwan. Detection by synthetic peptide-based assay and polymerase chain reaction. J. Hepatol. 17, 192–198.

Shiratori, Y., Kato, N., Yokosuka, O., Imazeki, F., Hashimoto, E., Hayashi, N., Nakamura, A., Asada, M., Kuroda, H., Tanaka, N., Arakawa, Y., Omata, M., 1997. Predictors of the efficacy of interferon therapy in chronic hepatitis C virus infection. Gastroenterology 113, 558–566.

Song, Z.Q., Hao, F., Min, F., Ma, Q.Y., Liu, G.D., 2001. Hepatitis C virus infection of human hepatoma cell line 7721 in vitro. World J. Gastroenterol. 7, 685–689.

Tagawa, M., Kato, N., Yokosuka, O., Ishikawa, T., Ohto, M., Omata, M., 1995. Infection of human hepatocyte cell lines with hepatitis C virus in vitro. J. Gastroenterol. Hepatol. 10, 523–527.

Tsuboi, S., Nagamori, S., Miyazaki, M., Mihara, K., Fukaya, K., Teruya, K., Kosaka, T., Tsuji, T., Namba, M., 1996. Persistence of hepatitis C virus RNA in established human hepatocellular carcinoma cell lines. J. Med. Virol. 48, 133–140.

Valli, M.B., Carloni, G., Manzi, A., Nasorri, F., Ponzetto, A., Clementi, M., 1997. Hepatitis C virus infection of a Vero cell clone displaying efficient virus-cell binding. Res. Virol. 148, 181–186.

World Health Organization, 1997. Hepatitis C: global prevalence. Wkly. Epidemiol. Rec. 10, 65–72.

Yoshikawa, M., Murakami, T., Hirano, K., Inadzuki, M., Ninomiya, K., Matsuda, H., 1997. Scorzonegosides A, B, and C, novel triterpene oligoglycosides with hepatoprotective effect from Chinese Bupleuri Radix, the roots of *Bupleurum scorzonerifolium* WILLD. Tetrahedron Lett. 38, 7395–7398.

Zibert, A., Schreier, E., Roggendorf, M., 1995. Antibodies in human sera specific to hypervariable region 1 of hepatitis C virus can block viral attachment. Virology 208, 653–661.

Ziegler, T., Hall, H., Sanchez-Fauquier, A., Gamble, W.C., Cox, N.J., 1995. Type- and subtype-specific detection of influenza viruses in clinical specimens by rapid culture assay. J. Clin. Microbiol. 33, 318–321.

Zoulim, F., Haem, J., Ahmed, S.S., Chossegros, P., Habersetzer, F., Chevallier, M., Bailly, F., Trepo, C., 1998. Ribavirin monotherapy in patients with chronic hepatitis C: a retrospective study of 95 patients. J. Viral Hepat. 5, 193–198.