

Figure 4. Effect of C-29EA on interferon signaling pathway. The Huh7 replicon cell line of genotype 1b was treated without (lane 3) or with 1, 10, 100, or 1000 U/mL interferon-alpha 2b (lanes 4–7), and 1.5 or 15 µg/ml C-29EA (lanes 1–2) for 48 h. Treatment with DMSO corresponds to '0'. The mRNAs of 2', 5'-OAS, and GAPDH as an internal control were detected by RT-PCR. Error bars indicate standard deviation. The data represent three independent experiments. doi:10.1371/journal.pone.0048685.g004

of replicon was decreased in the presence of C-29EA or interferon-alpha and further decreased by combination treatment using interferon-alpha and C-29EA (Fig. 8A). Furthermore, we employed the isobologram method [33] to determine whether antiviral effect of the combination treatment exhibits additive or synergistic. EC_{90} values of interferon-alpha and C-29EA were estimated at 10.7 U/ml and 26.4 µg/ml, respectively, in the absence of each other. EC_{90} values of C-29EA in the presence of 0, 2.5 and 5 U/ml interferon-alpha were plotted to generate an isobole. Figure 8B shows that the isobole exhibits concave

curvilinear, representing synergy but not additivity. These results suggest that combination treatment of interferon-alpha and C-29EA exhibits synergistic inhibition of HCV replication.

Discussion

Several natural products have been reported as anti-viral agents against HCV replication. Silbinin, epigallocatechin 3-gallate, and proanthocyanidins, which were prepared from milk thistle, green tea, and blueberry leaves, respectively, have exhibited inhibitory activity against HCV replication in cultured cells [34–37]. In our previous report, we identified manoalide as an anti-HCV agent from a marine sponge extract by high-throughput screening targeting NS3 helicase activity [38]. Manoalide inhibited ATPase, RNA binding, and NS3 helicase activity in enzymological assays. The EtOAc extract of the marine feather star also suppressed HCV replication in HCV replicon cell lines derived from genotype 1b, and it inhibited the RNA-binding activity but not the ATPase activity of NS3 helicase [30]. In this study, we screened 84 extracts of marine organisms for their ability to inhibit HCV replication in replicon cell lines and HCV cell culture system. Among these extracts, C-29EA, which was extracted from *Amphimedon* sp., most strongly inhibited HCV replication regardless of cytotoxicity. We previously reported that the EtOAc extract (SG1-23-1) of the feather star *Alloecomatella polycladia* inhibited HCV replication with an EC_{50} of 22.9 to 44.2 µg/ml in HCV replicon cells derived from genotype 1b [30]. Treatment with C-29EA potently inhibited HCV replication with an EC_{50} of 1.5 µg/ml and with an SI of more than 33.3 in the replicon cell line derived from genotype 1b, regardless of cytotoxicity (Fig. 1B and Table 2). However, C-29EA exhibited an EC_{50} of 24.9 µg/ml in a replicon cell line derived from genotype 2a at a weaker level than in the replicon cell line derived from genotype 1b (Figs. 1 and 2), suggesting that the ability of C-29EA to suppress HCV replication is dependent on the viral genotype or strain.

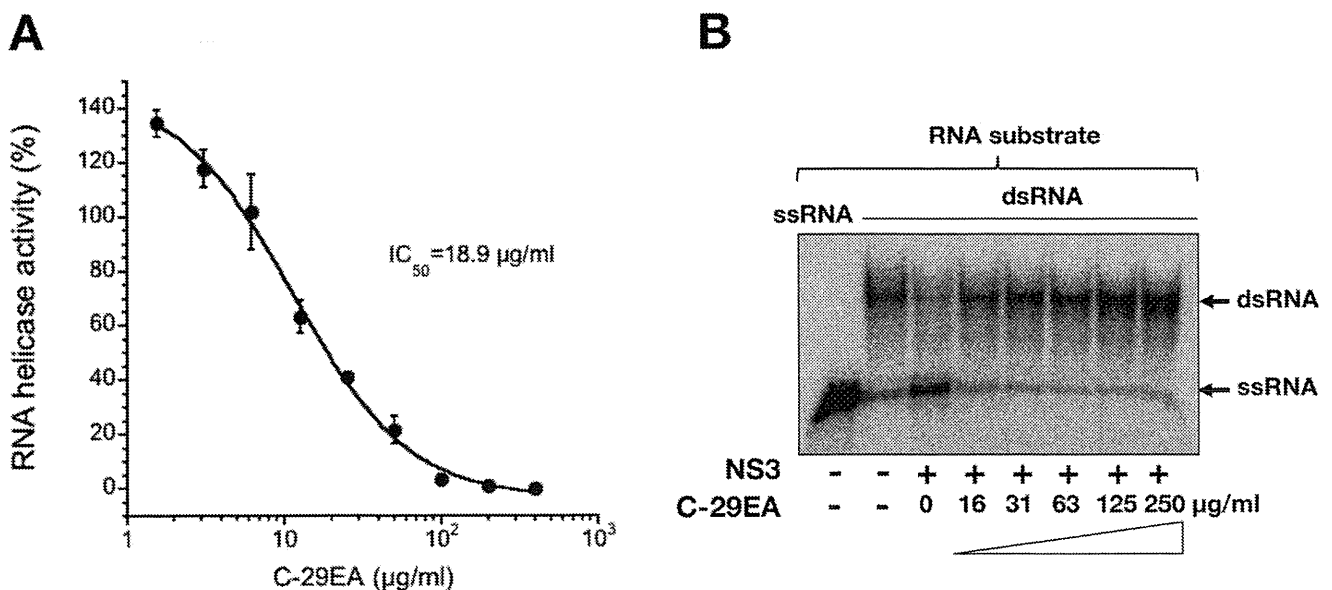


Figure 5. Effect of C-29EA on unwinding activity of NS3 helicase. (A) NS3 helicase activity was measured by PET assay. The reactions were carried out in the absence or presence of C-29EA. Helicase activity in the absence of C-29EA was defined as 100% helicase activity. Treatment with DMSO corresponds to '0'. The data are presented as the mean \pm standard deviation for three replicates. (B) The unwinding activity of NS3 helicase was measured by an RNA unwinding assay using radioisotope-labeled RNA. The heat-denatured single-strand RNA (26-mer) and the partial duplex RNA substrate were applied to lanes 1 and 2, respectively. The duplex RNA was reacted with NS3 (300 nM) in the presence of C-29EA (lanes 4–9, 16–250 µg/ml). The resulting samples were subjected to native polyacrylamide gel electrophoresis. Treatment with DMSO corresponds to '0'. doi:10.1371/journal.pone.0048685.g005

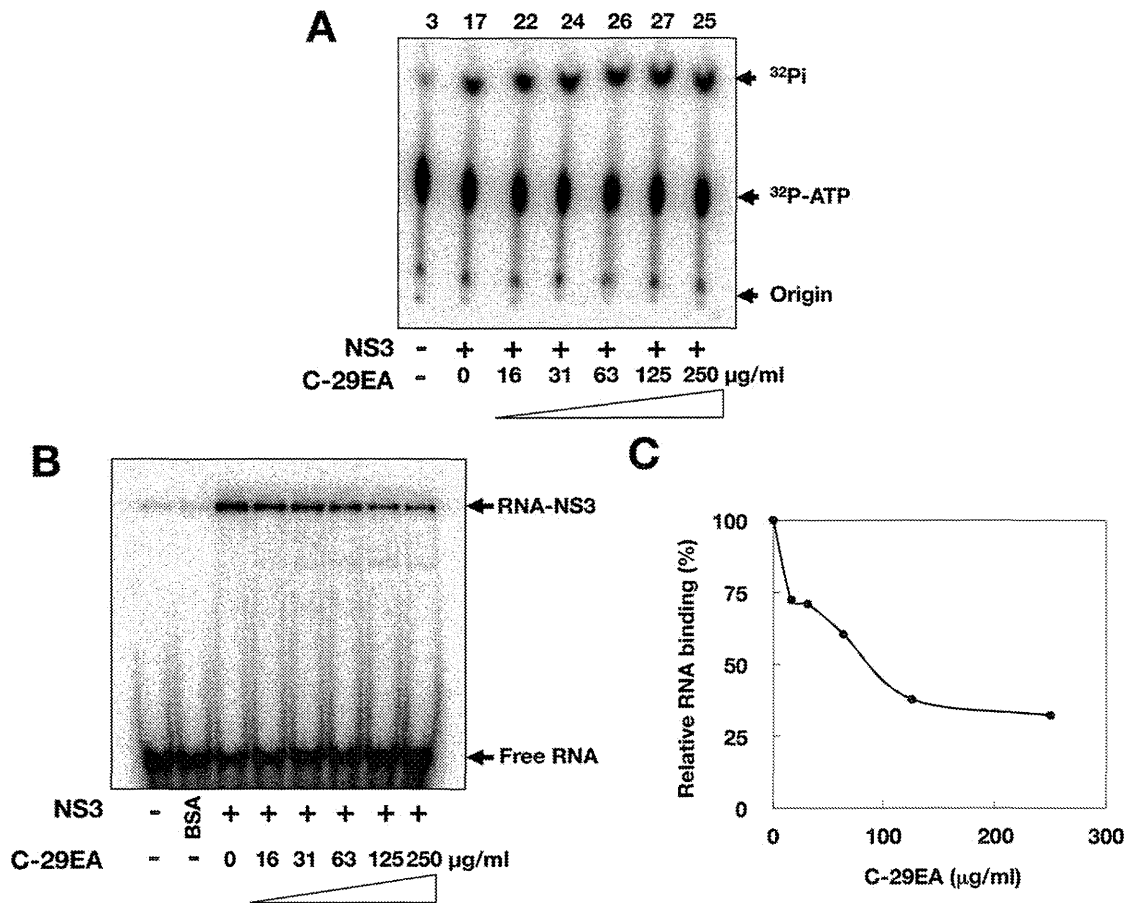


Figure 6. Effect of C-29EA on ATPase and RNA-binding activities of NS3 helicase. (A) The reaction mixtures were incubated with [γ - ^{32}P] ATP as described in Materials and Methods. The reaction mixtures were subjected to thin-layer chromatography. The start positions and migrated positions of ATP and free phosphoric acid are indicated as 'Origin', ' ^{32}P -ATP', and ' ^{32}P -Pi', respectively, on the right side of the figure. The data represent three independent experiments. Treatment with DMSO corresponds to '0'. (B) Gel mobility shift assay for RNA-binding activity of NS3 helicase. The reaction was carried out with 0.5 nM labeled ssRNA at the indicated concentrations of C-29EA or DMSO. The reaction mixture was subjected to gel mobility shift assay. (C) The relative RNA-binding ability was calculated with band densities in each lane and presented as a percentage of RNA-NS3 in the total density. The data represent three independent experiments. Treatment with DMSO corresponds to '0'. doi:10.1371/journal.pone.0048685.g006

HCV NS3 is well known to play a crucial role in viral replication through helicase and protease activities [5,39]. The N-terminal third of NS3 is responsible for serine protease activity in order to process the C-terminal portion of polyprotein containing viral nonstructural proteins [32]. The remaining portion of NS3 exhibits ATPase and RNA-binding activities responsible for helicase activity, which is involved in unwinding double-stranded RNA during replication of genomic viral RNA [40–42]. A negative-strand RNA is synthesized based on a viral genome (positive strand) after viral particles in the infected cells are uncoated, and is then used itself as a template to synthesize a positive-stranded RNA, which is translated or packaged into viral particles. Thus, both helicase and protease activities of NS3 are critical for HCV replication and could be targeted for the development of antiviral agents against HCV.

NS3 helicase activity was inhibited by treatment with C-29EA in a dose-dependent manner with an IC_{50} of 18.9 $\mu\text{g/ml}$ (Fig. 5A). RNA-binding activity, but not ATPase activity, was inhibited by treatment with C-29EA (Fig. 6). Treatment with C-29EA did not significantly affect the HCV-IRES activity and did not induce interferon-stimulated gene 2',5'-OAS (Figs. 3 and 4). Furthermore, the serine protease activity of NS3 was inhibited by using C-

29EA with an IC_{50} of 10.9 $\mu\text{g/ml}$ (Fig. 7). These results suggest that *Amphimedon* sp. includes the unknown compound(s) that could suppress NS3 enzymatic activity to inhibit HCV replication. Although the mechanism by which treatment with C-29EA could inhibit HCV replication has not yet been revealed, the unknown compound(s) may be associated with the inhibition of NS3 protease and helicase, leading to the suppression of HCV replication. However, other effects of extract C-29EA on HCV replication could not be excluded in this study.

The compound 1-N, 4-N-bis [4-(1H-benzimidazol-2-yl)phenyl] benzene-1,4-dicarboxamide, which is designated as (BIP) $_2$ B, was reported to be a potent and selective inhibitor of HCV NS3 helicase [43]. This compound competitively decreases the binding ability of HCV NS3 helicase to nucleic acids. The compound (BIP) $_2$ B inhibited RNA-induced stimulation of ATPase, although it did not directly affect the ATP hydrolysis activity of NS3 helicase. Thus, (BIP) $_2$ B could not affect ATPase activity without RNA or with a high concentration of RNA. Treatment with C-29EA inhibited helicase activity and viral replication but not ATPase activity (Figs. 1B, 2, 5, and 6). This extract suppressed the binding of RNA to helicase but exhibited no suppression of ATPase by NS3 helicase. Thus, the inhibitory action of extract C-29EA seems

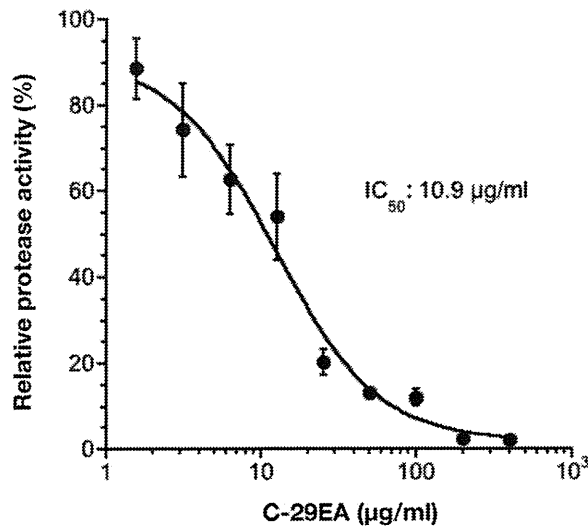


Figure 7. Effect of C-29EA on the activity of NS3 serine protease. NS3/4A serine protease was mixed with various concentrations of C-29EA or DMSO (0) in the reaction mixture and then incubated at 37°C for 120 min. The initial velocity at each concentration of C-29EA was calculated during 120 min reaction. The initial velocity in the absence of C-29EA was defined as 100% of relative protease activity. The data are presented as the mean \pm standard deviation for three replicates.

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different from that of (BIP)₂B. The quinolone derivative QU663 was reported to inhibit the unwinding activity of NS3 helicase by binding to an RNA-binding groove irrespective of its own ATPase activity [44]. The compound QU663 may competitively bind the RNA-binding site of NS3 but not affect ATPase activity, resulting in the inhibition of unwinding activity. In this study, treatment with C-29EA inhibited the RNA-binding activities of NS3 helicase but did not affect ATPase activity (Fig. 6). Furthermore, treatment with C-29EA suppressed the viral replication of HCV in an HCV cell culture system derived from several virus strains (Figs. 1 and 2, Table 2). The mechanism of C-29EA on the inhibition of NS3 helicase may be similar to that of compound QU663.

It is unknown whether one or several molecules included in C-29EA are critical for the inhibition of protease and helicase activities. The serine protease NS3/4A is one of the viral factors targeted for development into antiviral agents. Improvements in HCV therapy over the past several years have resulted in FDA approval of telaprevir (VX-950) [15,45] and boceprevir (SCH503034) [46,47]. Several studies suggest that the activities of NS3/4A protease and helicase in the full-length molecule enhance each other [48,49]. The NS3/4A protease has formed a complex with macrocyclic acylsulfonamide inhibitors [50,51]. Schiering et al. recently reported the structure of full-length NS3/4A in complex with a macrocyclic acylsulfonamide protease inhibitor [52], although the structure of full-length HCV NS3/4A in complex with a protease inhibitor has not been reported. The inhibitor binds to the active site of the protease, while the P4-capping and P2 moieties of the inhibitor are exposed toward the helicase interface and interact with both protease and helicase residues [52]. An unknown compound included in C-29EA might interact with both protease and helicase domains of NS3 to inhibit their activities. However, our data in this study have not excluded the possibility that several compounds included in C-29EA are related to the inhibition of protease and helicase of NS3/4A.

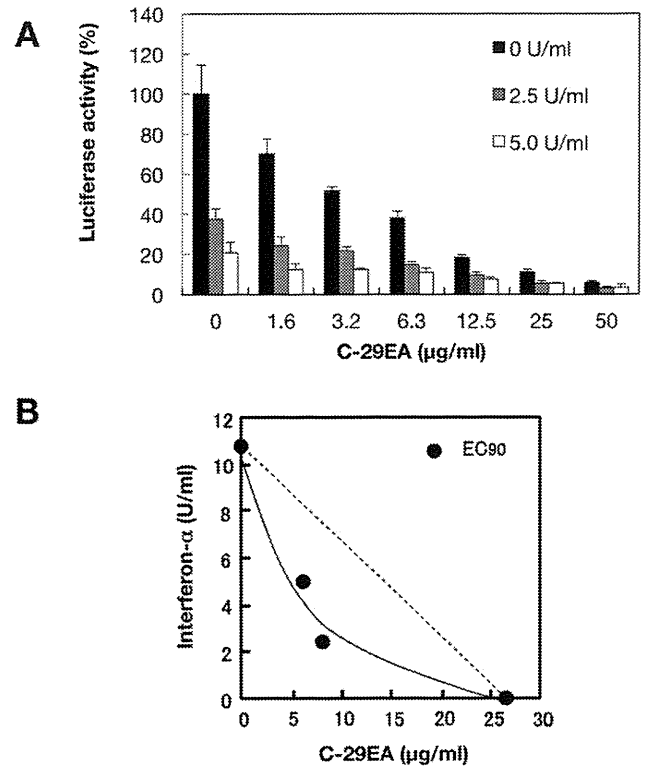


Figure 8. Effect of C-29EA on the antiviral activity of interferon-alpha. (A) The Huh7 cell line, including the subgenomic replicon RNA of genotype 1b strain Con1, was incubated in medium containing various concentrations of C-29EA or DMSO (0) in the presence or the absence of interferon-alpha. Luciferase assay were carried out as described in Materials and Methods. Error bars indicate standard deviation. The data represent three independent experiments. (B) Isobole plots of 90% inhibition of HCV replication. The broken line indicates the additive effect in the isobologram.

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In conclusion, we showed that the EtOAc extract from *Amphimedon* sp. significantly inhibits HCV replication by suppressing viral helicase and protease activities. The purification of an inhibitory compound from the extract of *Amphimedon* sp. will be necessary in order to improve its efficacy by chemical modification.

Materials and Methods

Preparation of Extracts from Marine Organisms

All marine organisms used in this study were hand-collected by scuba diving off islands in Okinawa Prefecture, Japan. No specific permits were required for the described field studies. We do not have to obtain a local government permit to collect invertebrates except for stony corals and marine organisms for fisheries, which we did not collect in this study. The areas where we collected are not privately-owned or protected in any way. We did not collect any invertebrates listed in the red data book issued by Ministry of Environment, Japan. The sponges, tunicates, and soft corals used in this study are not listed at all. Hence, no specific permits are required for this collection in the same way as the previous report of Aratake et al. [53].

The sponge from which C-29EA was extracted was identified as *Amphimedon* sp. and deposited at Naturalis under the code RMNH POR 6100. Each specimen was soaked in acetone. The acetone-extract fraction prepared from each specimen was concentrated.

The resulting material was fractionated as an EtOAc- and water-soluble fraction. The water-soluble fraction was dried up and solubilized in MeOH. The EtOAc- and the MeOH-soluble fractions were used for screening. All samples were dried and then solubilized in dimethyl sulfoxide (DMSO) before testing.

Cell Lines and Virus

The following Huh-7-derived cell lines used in this study were maintained in Dulbecco's modified Eagle's medium containing 10% fetal calf serum and 0.5 mg/ml G418. The Lunet/Con1 LUN Sb #26 cell line, which harbors the subgenomic replicon RNA of the Con1 strain (genotype 1b), was kindly provided by Ralf Bartenschlager [26]. Huh7/ORN3-5B #24 cell line, which harbors the subgenomic replicon RNA of the O strain (genotype 1b) was reported previously [54] and used for screening in this study (Table 1). HCV replicon cell line derived from genotype 2a strain JFH1 was described previously [55]. The surviving cells were infected with the JFH-1 virus at a multiplicity of infection (moi) of 0.05. The viral RNA derived from the plasmid pJFH1 was transcribed and introduced into Huh7OK1 cells according to the method of Wakita et al. [56]. The infectivity of the JFH1 strain was determined by a focus-forming assay [56].

Quantitative Reverse-transcription PCR (qRT-PCR) and Estimation of Core Protein

The estimation of viral RNA genome was carried out by the method described previously [57] with slight modification. Total RNAs were prepared from cells and culture supernatants by using an RNeasy mini kit (QIAGEN, Tokyo, Japan) and QIAamp Viral RNA mini kit (QIAGEN), respectively. First-strand cDNA was synthesized by using a high capacity cDNA reverse transcription kit (Applied Biosystems, Carlsbad, CA, USA) with random primers. Each cDNA was estimated by using Platinum SYBR Green qPCR SuperMix UDG (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Fluorescent signals of SYBR Green were analyzed by using an ABI PRISM 7000 (Applied Biosystems). The HCV internal ribosomal entry site (IRES) region was amplified using the primer pair 5'-GAGTGTCTGTCAGCCTCCA -3' and 5'-CACTCGCAAG-CACCCTATCA -3'. Expression of HCV core protein was determined by an enzyme-linked immunosorbent assay (ELISA) as described previously [57].

Determination of Luciferase Activity and Cytotoxicity in HCV Replicon Cells

HCV replicon cells were seeded at 2×10^4 cells per well in a 48-well plate 24 h before treatment. C-29EA was added to the culture medium at various concentrations. The treated cells were harvested 72 h post-treatment and lysed in cell culture lysis reagent (Promega, Madison, WI, USA) or *Renilla* luciferase assay lysis buffer (Promega). Luciferase activity in the harvested cells was estimated with a luciferase assay system (Promega) or a *Renilla* luciferase assay system (Promega). The resulting luminescence was detected by the Luminescencer-JNR AB-2100 (ATTO, Tokyo, Japan) and corresponded to the expression level of the HCV replicon. Cell viability was measured by a dimethylthiazol carboxymethoxy-phenylsulfophenyl tetrazolium (MTS) assay using a CellTiter 96 aqueous one-solution cell proliferation assay kit (Promega).

Effects on Activities of Internal Ribosome Entry Site (IRES)

Huh7 cells were transfected with pEF.Rluc.HCV.IRES.Feo or pEF.Rluc.EMCV.IRES.Feo and then were established in medium

containing 0.25 mg/ml G418, as described previously [58]. These cell lines were seeded at 2×10^4 cells per well in a 48-well plate 24 h before treatment, treated with 15 μ g/ml extract C-29EA, and then harvested at 72 h post-treatment. The firefly luciferase activities were measured with a luciferase assay system (Promega). The total protein concentration was measured using the BCA Protein Assay Reagent Kit (Thermo Scientific, Rockford, IL, USA) to normalize luciferase activity.

Western Blotting and Reverse-transcription Polymerase Chain Reaction (RT-PCR)

Western blotting was carried out by a method described previously [30]. The antibodies to NS3 (clone 8G-2, mouse monoclonal, Abcam, Cambridge, UK), NS5A (clone 256-A, mouse monoclonal, ViroGen, Watertown, MA, USA), and beta-actin were purchased from Cell Signaling Technology (rabbit polyclonal, Danvers, MA, USA) and were used as the primary antibodies in this study. RT-PCR was carried out by a method described previously [30,58].

Assays for RNA Helicase, ATPase, and RNA-binding Activities

A continuous fluorescence assay based on photoinduced electron transfer (PET) was described previously [29] and was slightly modified with regard to the reaction mixture [30]. The NS3 RNA unwinding assay was carried out by the method of Gallinari et al. [59] with slight modifications [30]. NS3 ATPase activity was determined by the method of Gallinari et al. [59] with slight modifications [30]. RNA binding to NS3 helicase was analyzed by a gel mobility shift assay [30,31]. The gene encoding NS3 helicase was amplified from the viral genome of genotype 1b and was introduced into a plasmid for the expression of a recombinant protein [38,60]. The radioactive band was visualized with the Image Reader FLA-9000 and quantified by Multi Gauge V 3.11 software.

NS3 Protease Assay

The fluorescence NS3 serine protease assay based on fluorescence resonance energy transfer (FRET) was carried out by the modified method using the SensoLyte™ 520 HCV protease assay kit (AnaSpec, Fremont, CA, USA). In brief, NS3 protein with a two-fold excess of NS4A cofactor peptide (Pep4AK) was prepared in $1 \times$ assay buffer provided with the kit. HCV NS3/4A protease was mixed with increasing concentrations of C-29EA and incubated at 37°C for 15 min. The reaction was started by adding the 5-FAM/QXL 520 substrate to the reaction mixture containing 180 nM HCV NS3/4A protease and various concentrations (0–400 μ g/ml) of C-29EA. The resulting mixture (20 μ l) was incubated at 37°C for 120 min using a LightCycler 1.5 (Roche Diagnostics, Basel, Switzerland). The fluorescence intensity was recorded every minute for 120 min. The NS3 serine protease activity was calculated as the initial reaction velocity and presented as a percentage of relative activity to that of the control examined with DMSO solvent but not C-29EA, in the same way as described in the fluorescence helicase assay [29].

Analysis of Drug-drug Interaction

The effects of drug combinations were evaluated using the isobologram method [33]. Various doses of C-29EA and interferon-alpha on 90% inhibition of HCV replication were combined to generate an isoeffect curve (isobole) to determine drug–drug interaction. Concave, linear, and convex curves exhibit synergy, additivity, and antagonism, respectively.

Statistical Analysis

The results are expressed as the mean \pm standard deviation. The significance of differences in the means was determined by Student's *t*-test.

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Author Contributions

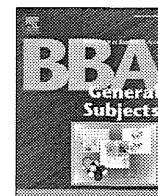
Conceived and designed the experiments: MN MT YS ST NA NN AY JT KM. Performed the experiments: YF KAS AF YM OF HT AY. Analyzed the data: MI NK NS SM NE. Wrote the paper: YF AY JT KM. Collected marine organisms: JT. Identified the sponge: NJdV.

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Different mechanisms of hepatitis C virus RNA polymerase activation by cyclophilin A and B in vitro

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ABSTRACT

Background: Cyclophilins (CyPs) are cellular proteins that are essential to hepatitis C virus (HCV) replication. Since cyclosporine A was discovered to inhibit HCV infection, the CyP pathway contributing to HCV replication is a potential attractive stratagem for controlling HCV infection. Among them, CyPA is accepted to interact with HCV nonstructural protein (NS) 5A, although interaction of CyPB and NS5B, an RNA-dependent RNA polymerase (RdRp), was proposed first.

Methods: CyPA, CyPB, and HCV RdRp were expressed in bacteria and purified using combination column chromatography. HCV RdRp activity was analyzed in vitro with purified CyPA and CyPB.

Results: CyPA at a high concentration (50× higher than that of RdRp) but not at low concentration activated HCV RdRp. CyPB had an allosteric effect on genotype 1b RdRp activation. CyPB showed genotype specificity and activated genotype 1b and J6CF (2a) RdRps but not genotype 1a or JFH1 (2a) RdRps. CyPA activated RdRps of genotypes 1a, 1b, and 2a. CyPB may also support HCV genotype 1b replication within the infected cells, although its knockdown effect on HCV 1b replicon activity was controversial in earlier reports.

Conclusions: CyPA activated HCV RdRp at the early stages of transcription, including template RNA binding. CyPB also activated genotype 1b RdRp. However, their activation mechanisms are different.

General significance: These data suggest that both CyPA and CyPB are excellent targets for the treatment of HCV 1b, which shows the greatest resistance to interferon and ribavirin combination therapy.

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1. Introduction

Hepatitis C virus (HCV¹), which belongs to the *Flaviviridae* family, has a positive-strand RNA genome, and its replication is regulated by viral and cellular proteins [1]. The genome encodes a large precursor polyprotein that is cleaved by host and viral proteases to generate at least 10 functional viral proteins: core, envelope 1 (E1), E2, p7, nonstructural protein 2 (NS2), NS3, NS4A, NS4B, NS5A, and NS5B [2]. NS5B is an RNA-dependent RNA polymerase (RdRp) [3–5].

Abbreviations: BSA, bovine serum albumin; CsA, cyclosporine A; CyP, cyclophilin; DTT, dithiothreitol; E, envelope; EDTA, ethylenediaminetetraacetic acid; GST, glutathione S-transferase; HCV, hepatitis C virus; NS, nonstructural protein; PPI, peptidyl prolyl *cis/trans*-isomerases; Peg-IFN, pegylated interferon- α ; PMSF, phenylmethanesulfonyl fluoride; RT-PCR, reverse transcription polymerase chain reaction; RdRp, RNA-dependent RNA polymerase; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis analysis; SVR, sustained virological response; Δ PPI, PPI knockout; wt, wild type

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HCV frequently establishes a persistent infection that leads to chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma [6,7]. More than 170 million individuals worldwide are infected with HCV [8], and the challenge of developing HCV treatment continues. First, combination therapy with pegylated interferon α (Peg-IFN α) and ribavirin led to a sustained virological response (SVR) in approximately 55% of patients infected with any HCV genotype and 42–46% of patients with genotype 1 [9,10]. However, many patients could not tolerate the serious adverse effects. Triple therapy consisting of an NS3/NS4A protease inhibitor (boceprevir or telaprevir), Peg-IFN (α -2a or α -2b), and ribavirin was then introduced, and it has become the standard regimen for genotype 1 infection. SVR improved significantly (from 63% to 75%), and the treatment duration decreased from 12 to 6 months [11,12]. However, triple therapy is more toxic than combination therapy [13].

Nonimmunosuppressant cyclosporine A (CsA) analogues/CyP inhibitors such as DEBIO-025 (Alisporivir) [14], NIM811 [15], and SCY-635 [16] are also the most expected candidates for use as anti-HCV drugs because their resistance selection is rare compared with other direct-acting antiviral agents, and the HCV resistant to

CyP inhibitors acquired mutations that allowed for reduced dependence on CyPs [17,18].

CyP was originally discovered as a cellular factor with high affinity for CsA [19]. CyPs comprise a family of peptidyl prolyl *cis/trans*-isomerases (PPI) that catalyze the *cis-trans* interconversion of peptide bonds amino terminal to proline residues, facilitating protein conformation changes [20]. CyPs are potential antiviral targets because CyPA was found to play a critical role in human immunodeficiency virus-1 infection [21,22]. The role of human CyPs as cellular cofactors in HCV replication was first suggested upon discovery of the anti-HCV effect of CsA [23–26]. Although the completion of a binding assay and the mapping of resistance initially suggested that NS5B was a viral target for CsA [27–29], recent papers have pointed to CyPA and NS5A as the central virus–host interaction involved in HCV replication [30–36]. Despite this unfavorable evidence, we analyzed the effect of CyPA and CyPB on HCV RdRp of various genotypes *in vitro* and found differences in genotype specificity and the mechanism of HCV RdRp activation.

2. Materials and methods

2.1. Purification of HCV RdRp

HCV RNA RdRps with C-terminal 21 amino acid deletion of 1a (H77 and RMT), 1b (HCR6, NN, and Con1), and 2a (JFH1 and J6CF) were expressed in *E. coli* Rosetta/pLysS and purified as described previously [37–40]. The purified HCV RdRps (5 μ M, >95% pure) were stocked in 20 mM Tris–HCl (pH 8.0), 500 mM NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA), 5 mM dithiothreitol (DTT), 5% glycerol, and 1 mM phenylmethanesulfonylfluoride (PMSF) at -80°C . The yield of HCV RdRps is approximately 1.7 mg from a 1-L bacterial culture. The purified HCV RdRps were as shown in Fig. S1 of Weng et al. [38]. The protein purities were determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis analysis (SDS-PAGE), using ImageJ 1.46 (<http://rsbweb.nih.gov/ij/>).

2.2. Construction of CyP-expressing plasmids

Human CyPA and CyPB were cloned from total RNA extracted from 293T cells, using a reverse transcription-polymerase chain reaction (RT-PCR) kit (Takara, Dalian, China) as published previously [29]. After being digested with *Bam*HI and *Eco*RI, they were cloned into the same site of pGEX-6P-3 (GE Healthcare, Bucks, UK), resulting in pGEXCyPA and pGEXCyPB, respectively. CyPB Δ PPI, the enzymatic inactive mutant of CyPB, was PCR cloned into pGEX-6P-3 from pCMV-CyPB Δ PPIFL [29], resulting in pGEXCyPB Δ PPI. CyPA Δ PPI was produced by the introduction of the R55A and F60A mutations using a QuickChange Site-Directed Mutagenesis Kit (Stratagene, St. Clara, CA, USA) and primers (5'-GTTCTGCTTTCACGCCATTATCCAGGGG CCATGTGTCAGGGTG-3' and 5'-CACCTGACACATGGCCCTGGAATAA TGGCGTGAAAGCAGGAAC-3').

2.3. Purification of CyPs

E. coli Rosetta were transformed using pGEXCyPA, pGEXCyPA Δ PPI, pGEXCyPB, and pGEXCyPB Δ PPI. GST-tagged CyPA, CyPB, CyPA Δ PPI, and CyPB Δ PPI were induced with 1 mM isopropyl β -D-1-thiogalactopyranoside at 18°C for 4 h. The bacteria were harvested and stocked at -20°C . After thawing on ice, the bacteria were lysed in 4 packed cell volumes of phosphate-buffered saline, 0.1% Triton X-100, 1 mM EDTA, 1 mM DTT, and 1 mM PMSF. After being clarified by centrifugation at 10,000 \times g for 30 min at 4°C and filtered through a 0.45- μ m nitrocellulose filter, the extract was incubated with Glutathione Sepharose 4B (GE Healthcare) for 30 min at 4°C . After the resin was washed with 50 mM Tris–HCl (pH 8.0), 500 mM NaCl, 1 mM EDTA, 1 mM DTT, and 1 mM PMSF, the GST-CyP was eluted using 50 mM Tris–HCl (pH 8.0), 500 mM NaCl,

1 mM EDTA, 1 mM DTT, 10 mM reduced glutathione, and 1 mM PMSF, followed by gel filtration through a Superdex 200 column (GE Healthcare) in 20 mM Tris–HCl (pH 8.0), 500 mM NaCl, 1 mM EDTA, 1 mM DTT, and 10% glycerol. The eluted GST-CyP were diluted to 50 mM NaCl and applied to a MonoQ (GE Healthcare) in 20 mM Tris–HCl (pH 9.0), 50 mM NaCl, 1 mM EDTA, 1 mM DTT, and 10% glycerol. GST-CyPB and GST-CyPB Δ PPI were chromatographed using a continuous NaCl gradient of 50–1000 mM. The purified CyPs were stocked at -20°C .

2.4. *In vitro* HCV transcription with CyPs

In vitro HCV transcription with CyPs was done as previously described [37–40]. Briefly, the indicated amounts of the CyPs were incubated in 50 mM Tris–HCl (pH 7.5), 200 mM monopotassium glutamate, 3.5 mM MnCl₂, 1 mM DTT, 0.5 mM GTP, 200 nM of a 184-nt *in vitro* transcribed model RNA template (SL12-1S), 100 U/mL of human placental RNase inhibitor, and 100 nM HCV RdRp at 29°C for 30 min. After preincubation, RdRp was incubated for an additional 90 min with 50 μ M ATP, 50 μ M CTP, or 5 μ M [α -³²P]UTP. The RNA products were analyzed using 6% PAGE containing 8 M urea after being purified by phenol/chloroform extraction and ethanol precipitation. The amount of RNA products was analyzed using Typhoon Trio (GE Healthcare).

2.5. RNA filter-binding assay with CyPA and CyPB

An RNA filter-binding assay with CyPA and CyPB was performed as previously described [37,38,40]. Briefly, [³²P]-SL12-1S was incubated in 25 μ L of 50 mM Tris–HCl (pH 7.5), 200 mM monopotassium glutamate, 3.5 mM MnCl₂, 1 mM DTT, and 5 pmol of HCV RdRp with 375 pmol (75 \times) of CyPA and 25 pmol (5 \times) of CyPB at 29°C for 30 min.

2.6. Chemicals and radioisotopes

[α -³²P]UTP (800 Ci/mmol, 40 mCi/mL) was purchased from PerkinElmer Life Sciences (Waltham, MA, USA). The nucleotides were purchased from GE Healthcare. The human placental RNase inhibitor T7 RNA polymerase and PrimeSTAR HS DNA polymerase were purchased from Takara. The bacteria were purchased from Novagen (Merck Chemicals, Darmstadt, Germany).

2.7. Statistical analysis

The statistical data were evaluated using Student's *t* test, with $p < 0.05$ indicating statistical significance.

3. Results

3.1. Purification of CyPA and B

First, glutathione S-transferase (GST)-tagged CyPA, CyPB, the PPI inactive CyPA (CyPA Δ PPI), and CyPB (CyPB Δ PPI) were purified using Glutathione Sepharose 4B affinity chromatography. CyPA and CyPA Δ PPI were further purified through a Superdex 200 column (Fig. S1). After the Superdex 200 gel filtration, to remove the contaminating nucleic acids, CyPB and CyPB Δ PPI were further purified through MonoQ anion exchange chromatography by a continuous NaCl gradient of 50–1000 mM because CyPB has a strong affinity for nucleic acids. Each was eluted with 210–385 mM NaCl (Fig. S2). The purification scheme and purified CyPs are shown in Fig. 1. The yields of CyPA and CyPA Δ PPI were approximately 3 mg from a 1-L bacterial culture. CyPA and CyPA Δ PPI were >95% pure and stocked at 5 mg/mL in 20 mM Tris–HCl (pH 8.0), 500 mM NaCl, 1 mM EDTA, 1 mM DTT, and 10% glycerol. CyPB and CyPB Δ PPI were stocked at 5 mg/mL in 20 mM Tris–HCl (pH 9.0), 500 mM NaCl, 1 mM EDTA, 1 mM DTT,

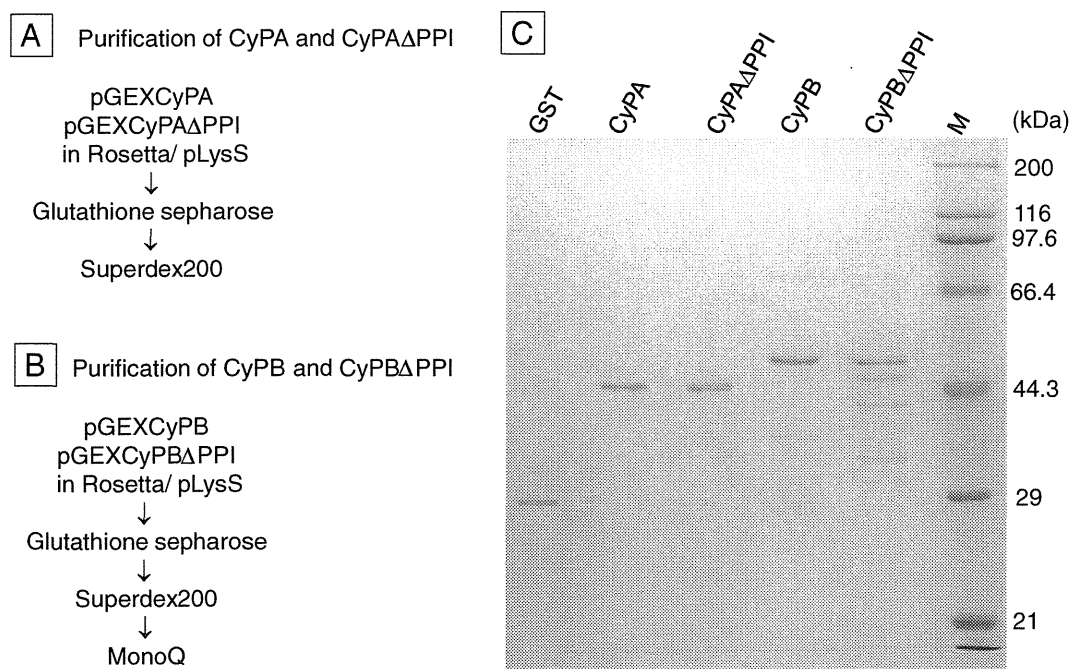


Fig. 1. Cyclophilin purification. The purification schemes of cyclophilin A (CyPA) and the peptidyl prolyl isomerase-inactive mutant protein of CyPA (CyPA Δ PPI) (A), cyclophilin B (CyPB) and CyPB Δ PPI (B), and sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) (C) with 5 pmol each of purified glutathione S-transferase (GST; 28.3 kDa), GST–CyPA (44.9 kDa), GST–CyPA Δ PPI (44.7 kDa), GST–CyPB (52.1 kDa), and GST–CyPB Δ PPI (52 kDa) were separated through 10% SDS–PAGE and stained with Coomassie brilliant blue. The sizes of the molecular weight standards (M) are indicated on the right side of the gel. Their final elution profiles are shown in Figs. S1 and S2.

and 10% glycerol. The yields of CyPB and CyPB Δ PPI were approximately 1 mg from a 1-L bacterial culture. The purities of CyPB and CyPB Δ PPI were >95% and >90%, respectively.

3.2. HCV 1b and JFH1 (2a) transcription in vitro with CyPA and CyPB

The dose–response effects of CyPA and CyPB were examined using an in vitro transcription system of HCR6 (1b) and JFH1 (2a) RdRp wild type (wt). CyPA and CyPB were added to the optimal HCV in vitro transcription condition while the RNA synthesis was in the log phase [4,37]. RdRp (100 nM) was incubated with 0, 50 (ratio to RdRp: 0.5 \times), 100 (1 \times), 200 (2 \times), 500 (5 \times), and 1000 nM (10 \times) CyPA and CyPB, GST, or bovine serum albumin (BSA) in GTP (the initiating nucleotide) and an RNA template for 30 min, followed by elongation with ATP, CTP, and UTP for 90 min. CyPA enhancement was further tested using 2 (20 \times), 5 (50 \times), 7.5 (75 \times), and 10 (100 \times) μ M because the enhancement effect of CyPA under 1 μ M (10 \times) was unclear. Fig. S3 shows the autoradiography of HCV HCR6 (1b) and JFH1 (2a) RdRpwt with CyPA and CyPB, the graphs of which were drawn using the data from 3 independent experiments (Fig. 2).

The CyPA activation of both RdRps showed 2 reaction speeds. The first-order ratio of CyPA to HCR6 (1b) RdRpwt <50 \times is fitted as a linear regression curve, the equation for which is $y = 0.07x$ (CyPA-to-RdRp ratio) + 0.7. The linear regression curve fitting of the ratio >50 \times is $y = 0.4x$ (CyPA-to-RdRp ratio) – 17 when calculated from 3 points. That of CyPA to JFH1 (2a) RdRpwt is fitted to a similar linear regression, $y = 0.09x$ (CyPA-to-RdRp ratio) + 0.9 (the CyPA-to-RdRp ratio <50 \times). HCR6 (1b) and JFH1 (2a) RdRps were activated by 100 \times CyPA to 25 \pm 0.2- and 19 \pm 1-fold, respectively.

The CyPB activation of HCR6 (1b) RdRpwt occurred in a dose-dependent manner and fitted a sigmoid curve, and the enhancement effect reached a plateau (9.4 \times) at the ratio of 5 \times . Neither GST nor BSA enhanced HCR6 (1b) RdRpwt. CyPB, GST, and BSA did not enhance JFH1 (2a) RdRpwt (<1.5 \times) at the concentrations described earlier.

3.3. Effect of the PPI inactive mutant proteins of CyPA and CyPB

CyP has PPI activity. To test the contribution of PPI activity to HCV HCR6 (1b) and JFH1 (2a) RdRpwt activation, the activation effect of the PPI inactive mutant proteins, CyPA Δ PPI at 100 \times (10 μ M) and CyPB Δ PPI at 2 \times (200 nM), were tested together with 100 \times (10 μ M) GST and BSA (Fig. 3). CyPA enhanced JFH1 (2a) RdRpwt 17.6 \times , whereas CyPA Δ PPI enhanced it 16.2 \times . This difference is statistically significant (Student's *t* test, $p < 0.05$). CyPA enhanced HCR6 (1b) RdRpwt activity 27.7 \times , whereas CyPA Δ PPI enhanced it 16.0 \times . BSA slightly inhibited both RdRps at the same concentration in this experiment. As shown in Fig. 2C and D, it can be concluded that BSA has no effect on HCV transcription. GST enhanced JFH1 (2a) RdRpwt activity 5.0 \times , but it did not affect HCR6 (1b) RdRpwt activity. CyPB enhanced HCR6 (1b) RdRpwt activity 2.3 \times , whereas CyPB Δ PPI enhanced it 1.7 \times . This difference is also statistically significant (Student's *t* test, $p < 0.05$). JFH1 (2a) RdRpwt was not activated by CyPB or CyPB Δ PPI.

3.4. CyP activation steps of HCV transcription

The HCV transcription steps of CyP enhancement were analyzed by the sequential addition of CyPs during in vitro transcription (Fig. 4). CyPA enhanced HCR6 (1b) and JFH1 (2a) RdRpwt, whereas CyPB enhanced HCR6 (1b) RdRpwt when HCV RdRps were incubated with them from the start of transcription (initiation). The CyP effect was then tested after their addition during the elongation period after HCV RdRps was initiated with GTP. CyPA (100 \times ; 10 μ M) and CyPB (5 \times ; 500 nM) were added to HCV RdRps after the 30-min incubation with GTP, when 3 GTPs were incorporated at the 5' end of the products. CyPB did not enhance HCR6 (1b) or JFH1 (2a) RdRp when added during the elongation period, although it enhanced HCV RdRp when added at the start of transcription. CyPA enhanced HCR6 (1b) and JFH1 (2a) RdRp activity only 1.6 \times (Student's *t* test, $p < 0.05$) and 2.1 \times ($p < 0.01$), respectively, when added during the elongation step. These results suggest that CyPA and CyPB activated only the transcription initiation step of HCV RdRps.

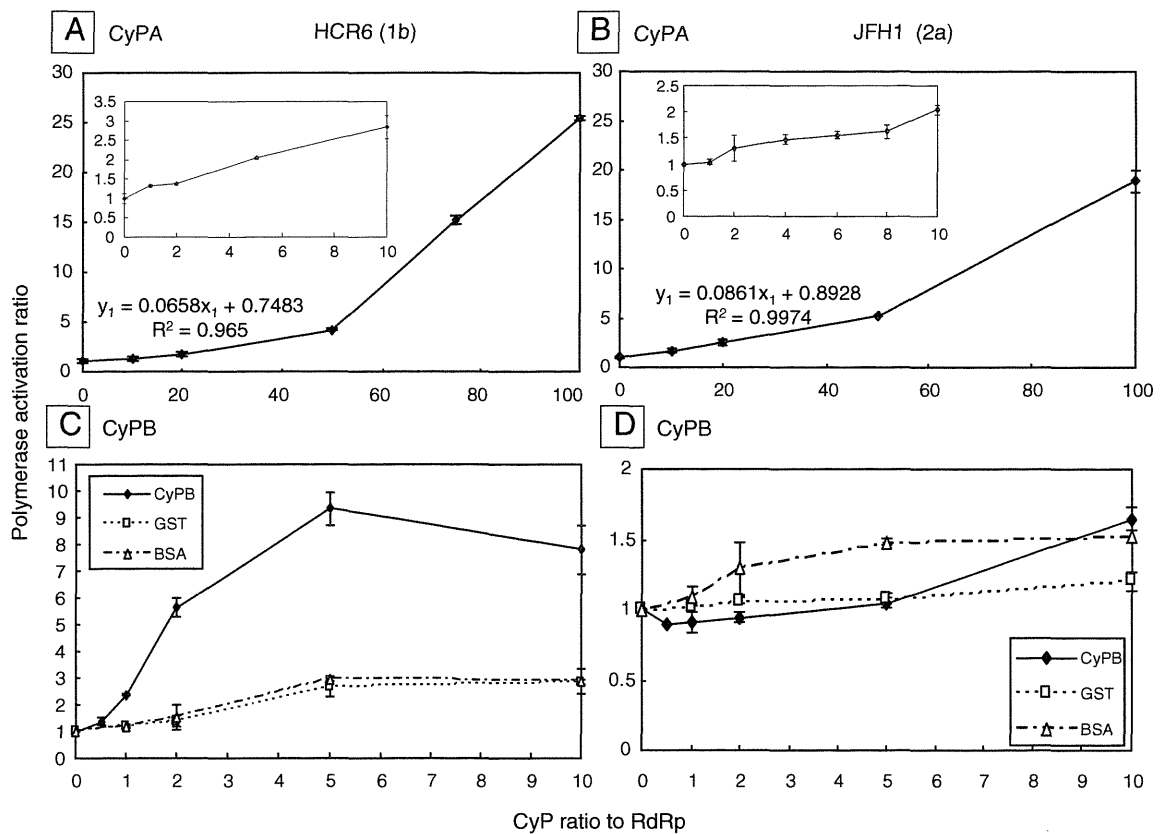


Fig. 2. Dose–response curve of cyclophilin A (CyPA) and cyclophilin B (CyPB) in hepatitis C virus (HCV) transcription in vitro. The dose–response curve of the HCV RdRp activation of CyPA in HCR6 (1b) RdRpwt (A) and JFH1 (2a) RdRpwt (B) CyPB in HCR6 (1b) RdRpwt (C) and JFH1 (2a) RdRpwt was drawn from the image analysis of Fig. S3. Insets A and B indicate that of 0, 0.5×, 1×, 2×, 5×, and 10× of CyPA to RdRp. The first-order ratio of the curves of A and B were fit by linear regression, and the calculated equations are indicated in the graph. The mean relative polymerase activation ratio and standard deviation (error bar) were calculated from 3 independent measurements.

The effects of 75× CyPA and 5× CyPB on the RNA-binding activity of HCR6 (1b) and JFH1 (2a) RdRp were then tested (Fig. 4E). The effects of HCR6 (1b) and JFH1 (2a) RdRp with CyPA were 10.1 ± 0.56- and 6.6 ±

0.68-fold of that without CyPA, respectively. The effect of HCR6 (1b) RdRp with CyPB was 3.1 ± 0.3-fold of that without CyPB. The RNA-binding activity of HCV RdRps was thus enhanced by the addition of CyPA and CyPB.

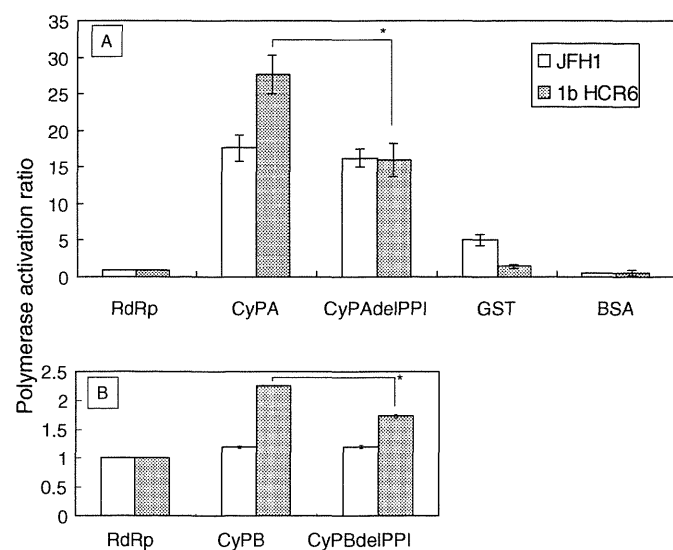


Fig. 3. Effects of cyclophilin A (CyPA) and cyclophilin B (CyPB) with and without peptidyl prolyl isomerase activity on hepatitis C virus (HCV) JFH1 (2a) and HCR6 (1b) RdRp. HCV HCR6 (1b) and JFH1 (2a) RdRpwt (100 nM) were incubated with 100× (10 μM) of CyPA, CyPAΔPPI, glutathione S-transferase (GST), and bovine serum albumin (BSA) (A). HCV RdRps were incubated with 5× (500 nM) of CyPB, CyPBΔPPI, GST, and BSA (B). The mean relative polymerase activity and standard deviation (error bar) were calculated from 3 independent measurements. *p < 0.01 (Student's *t* test).

3.5. Effect of CyP activation on RdRp of various HCV genotypes

The CsA sensitivity differed among the HCV genotypes [41]. Therefore, we tested the effects of CyPA and CyPB activation on NN (1b), H77 (1a), RMT (1a), and J6CF (2a) RdRp (Fig. 5). RdRp activity was compared with and without 50× (5 μM) CyPA and 5× (500 nM) CyPB. At their respective concentrations, CyPA activated all of the tested HCV RdRps by 3.9–5.3×, but CyPB activated only 1b RdRps (8–10×). CyPB slightly activated J6CF (2a) RdRp (approximately 4×), but it did not activate the 1a or JFH1 (2a) RdRps (1.4–1.8×).

4. Discussion

Since CsA was discovered to inhibit HCV infection [23–26], the CyP pathway contributing to HCV replication has been proposed as a potential stratagem for controlling HCV infection. Reports about the roles of CyPA in HCV replication via NS5A have been accumulating [33–35,42–44]. However, the effect of CyP inhibitors varied on the RNA-binding activity of NS5B [41,45], and DEBIO-025 decreased CyPB levels in patients [46]. Controversial results of CyPA and CyPB knockout experiments on HCV replicon activity were reported [29,30,47]. Therefore, the effects of CyPA and CyPB on HCV RdRp were carefully analyzed again in vitro.

In this study, we demonstrated that CyPA and CyPB activated HCV 1b RdRp in vitro by completely different kinetics using purified CyPs

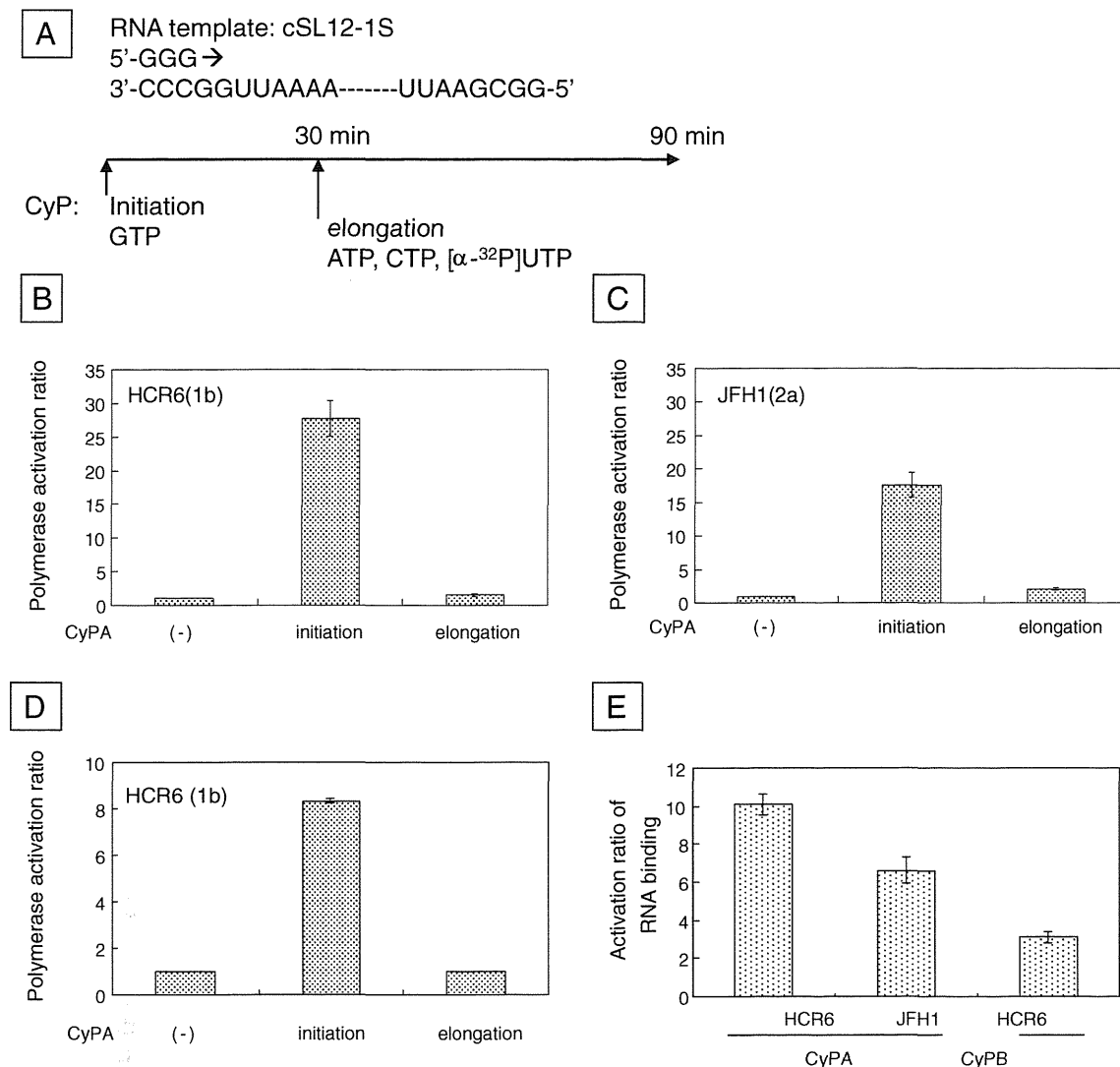


Fig. 4. Hepatitis C virus (HCV) RdRp activation effects of cyclophilin A (CyPA) and cyclophilin B (CyPB) on transcription initiation and elongation. The polymerase activation effect of the timing of the CyPA or CyPB addition was examined. The sequence of the model RNA template (SL12-1S) and experimental design are shown in A. CyPA 100 \times (10 μ M) was incubated with HCR6 (1b) RdRpwt (A) and JFH1 (2a) (B) RdRp during preincubation with 0.5 mM GTP (initiation) or after preincubation (elongation). CyPB 5 \times (500 nM) was incubated with HCR6 (1b) RdRpwt during preincubation with 0.5 mM GTP (initiation) or after the preincubation (elongation) (C). The mean relative polymerase activation ratio and standard deviation (error bar) were calculated from 3 independent measurements. The effect of the 100 \times CyPA and 5 \times CyPB on RNA template binding was examined (E).

and HCV RdRps (Fig. 2), which indicated that the mechanism of their HCV RdRp activation differed despite their similar structures [48–50]. Kinetic analysis of CyPA on HCR6 (1b) and JFH1 (2a) RdRp indicated that it had a similar activation mechanism on both HCV RdRps. CyPA did not activate HCV RdRp at low concentrations, but it did activate it at $>50\times$ molar excess to it. The unusual dose of CyPA activating HCV RdRp (Fig. 2) postulates that HCV RdRp may be surrounded by CyPA in vitro and factors involving CyPA and HCV RdRp interaction, such as NSSA, in the HCV replication complex of the infected cells [27,28,31,36,51–53] because the interaction of CyPA and HCV RdRp was weak (Fig. S4).

Although some controversial results were obtained from those of Heck et al. [54], the studies agree that CyPB also activated HCV 1b RdRp in vitro. The activation kinetics of CyPB on HCR6 (1b) RdRp showed a sigmoid-like curve (Fig. 2) that suggested an allosteric effect of CyPB on RdRp activity. CyPB may interact with HCV RdRp as a cofactor and directly activate HCR6 (1b) RdRp. The HCV RdRp–CyPB complex was likely to interact more with CyPB, and its activation plateaued at the CyPB/RdRp ratio of 5:1 (Fig. 2C). The CyPB

activation curves of Heck et al. [54] also plateaued. These data from the 2 independent groups support the weak interaction between CyPB and HCV 1b RdRp (Fig. S4).

CyPA did not show genotype specificity in the current study (Fig. 5A), a finding that agrees with those of CyPA knockdown, DEBIO-025, and CsA experiments [30,43,55]. CyPB activation showed genotype specificity (Fig. 5B) [54]; CyPB activated 1b and J6CF (2a) RdRp but did not activate 1a or JFH1 (2a) RdRp. Both reports agreed with the finding that JFH1 (2a) subgenomic replicon was independent of CyPB [41]. Although mutations accumulated in the NS5A region of CsA- or DEBIO-025-resistant HCV replicons, some mutations were found in the NS5B region [18,27,28,33,45].

Another controversial result between that of Heck et al. [54] and ours is the Mg^{2+} -dependency of the CyPB activation. The Mg^{2+} concentration in cells is 14–20 mM, and Mg^{2+} ions are distributed almost equally throughout the nuclei, mitochondria, and cytosol/endoplasmic reticulum [56]. The Mn^{2+} concentration in cells varies from report to report [57,58]. The optimal Mn^{2+} and Mg^{2+} concentrations in the HCV in vitro transcription used in this study were

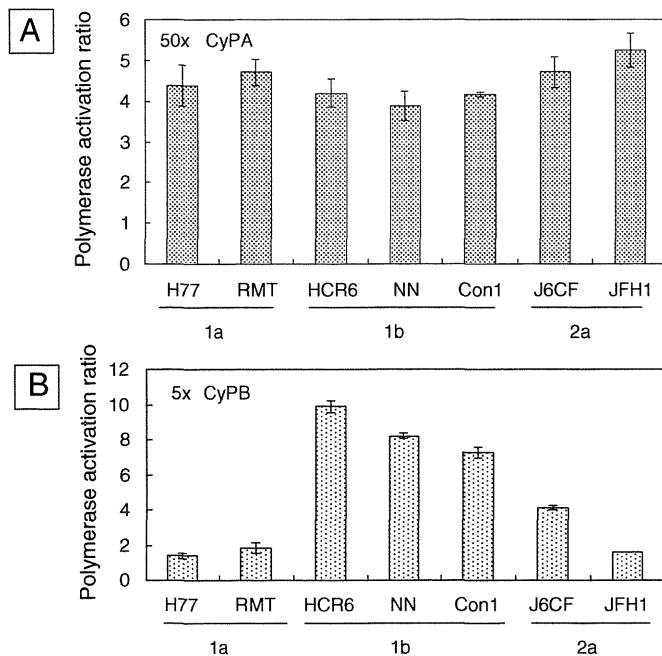


Fig. 5. Activation effects of cyclophilin A (CyPA) and cyclophilin B (CyPB) on hepatitis C virus (HCV) RNA polymerase of genotypes 1a, 1b, and 2a. The polymerase activation effects of CyPA and CyPB on HCV 1a (H77 and RMT), 1b (HCR6, NN, and Con1), and 2a (J6CF and JFH1) were examined. HCV RdRp (100 nM) was incubated with 50× CyPA and 5× CyPB. The mean relative polymerase activation ratio and standard deviation (error bar) were calculated from 3 independent measurements.

different from the physiological concentrations in cells [4,37]. However, under the optimal HCV transcription condition, HCV RdRp activation was observed by CyPA and CyPB (Fig. 1).

The amount of CyPA varies by cell type [59]. In some cells, CyPB may also contribute to HCV 1b replication because it localizes in the endoplasmic reticulum and plasma membranes [60,61], which form a membrane web in which an HCV replication complex exists [1].

PPI activity of CyPs is essential for HCV replicon activation [32,53]. CyP inhibitors (DEBIO-025, NIM811, and SCY-635) inhibit PPI activity. The PPI activity of CyPA contributed to HCV RdRp activation and CyP-NS5A binding [36]. The PPI activity of CyPA partly contributed to the activation of HCR6 (1b) RdRpwt in vitro (Fig. 3A, $p < 0.01$). The PPI activity of CyPB may not be essential for RdRp activation because the activation ratio was not large between CyPB and CyPBΔPPI, although the experiment showed a statistically significant difference (Fig. 3B). There may be differences in the RdRp activation mechanisms of CyPA with and without PPI activity. This finding will help with the development of new CyPA inhibitors that target domains other than PPI.

The mechanism of HCV RdRp activation by CyPs is not clear. In the least, CyPA and CyPB enhanced the early stage of HCV transcription, including the template RNA binding of HCV RdRp (Fig. 4) [29,41,45]. The productive template-polymerase binding is the late-limiting step of transcription initiation by HCV RdRp in vitro, and a small fraction of HCV RdRp was active in vitro [62,63]. CyP may enhance this step on many HCV RdRp molecules to show apparent activation of RdRp in vitro.

Considering the controversial reports on CyP and HCV replication [29,33,35,41,43,44], it can be concluded that CyPA is the major factor of HCV genome replication and that the activation of HCV RdRp may require other factors such as NS5A to condense CyPA around the HCV RdRp. Although many HCV treatment approaches have been applied in addition to Peg-IFN, ribavirin, and NS3/NS4a protease inhibitor [64–67], more effort has to be made to ensure an HCV cure. This

study and that of Heck et al. [54] demonstrated similar activation kinetics and genotype specificity of CyPB activation (Figs. 2 and 5). CyPB also has the potential to activate HCV 1b genome replication in a limited condition, and it should also be included as the target of inhibitor development because HCV 1b is the genotype that is most resistant to treatment [13].

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.bbagen.2012.08.017>.

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Japanese Reference Panel of Blood Specimens for Evaluation of Hepatitis C Virus RNA and Core Antigen Quantitative Assays

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An accurate and reliable quantitative assay for hepatitis C virus (HCV) is essential for measuring viral propagation and the efficacy of antiviral therapy. There is a growing need for domestic reference panels for evaluation of clinical assay kits because the performance of these kits may vary with region-specific genotypes or polymorphisms. In this study, we established a reference panel by selecting 80 donated blood specimens in Japan that tested positive for HCV. Using this panel, we quantified HCV viral loads using two HCV RNA kits and five core antigen (Ag) kits currently available in Japan. The data from the two HCV RNA assay kits showed excellent correlation. All RNA titers were distributed evenly across a range from 3 to 7 log IU/ml. Although the data from the five core Ag kits also correlated with RNA titers, the sensitivities of individual kits were not sufficient to quantify viral load in all samples. As calculated by the correlation with RNA titers, the theoretical lower limits of detection by these core Ag assays were higher than those for the detection of RNA. Moreover, in several samples in our panel, core Ag levels were underestimated compared to RNA titers. Sequence analysis in the HCV core region suggested that polymorphisms at amino acids 47 to 49 of the core Ag were responsible for this underestimation. The panel established in this study will be useful for estimating the quality of currently available and upcoming HCV assay kits; such quality control is essential for clinical usage of these kits.

Hepatitis C virus (HCV) is a major cause of chronic liver disease worldwide (15). There is no protective vaccine against this virus, and once an individual is infected, HCV often establishes persistent infection and leads to chronic hepatitis, cirrhosis, and hepatocellular carcinoma (9). The most widely used therapy for HCV infection is the combined administration of pegylated alpha interferon and ribavirin (29). However, this treatment is problematic, as it has limited efficacy, high cost, and severe adverse effects (8, 25). To estimate the outcome of antiviral therapy, and to understand the state of viral propagation, it is important to determine the HCV viral load in chronic hepatitis C patients by the use of accurate and reliable HCV quantitative assays (9, 14). For this purpose, several commercial assay kits for HCV RNA and core antigen (Ag) quantification are currently used in Japan. For quantification of HCV RNA levels, two real-time quantitative reverse transcription-PCR (qRT-PCR)-based assay kits are available, including the COBAS AmpliPrep/COBAS TaqMan HCV test (CAP/CTM-RNA; Roche Diagnostics, Tokyo, Japan) and the Abbott RealTime HCV test (ART-RNA; Abbott Japan, Tokyo, Japan). These assays are known to have high sensitivity and a wide dynamic range, but they require technical skill and attention to maintaining the specified conditions (4–6, 16, 24, 33–35). Alternatively, HCV viremia can be quantified by assessment of HCV core Ag level (1–3, 7, 10, 12, 13, 17–22, 27, 30–32). Five HCV core Ag assay kits are commercially available in Japan, including Architect HCV Ag (Architect-Ag; Abbott Japan), Lumipulse Ortho HCV Ag (Lumipulse-Ag; Fujirebio, Tokyo, Japan), Lumispot Eiken HCV Ag (Lumispot-Ag; Eiken Chemical, Tokyo, Japan), the Ortho HCV Ag ELISA test (ELISA-Ag; Ortho Clinical Diagnostics, Tokyo, Japan), and the Ortho HCV Ag IRMA test (IRMA-Ag; Ortho Clinical Diagnostics, Tokyo, Japan). These assays have some disadvantages compared to those measuring HCV RNA (notably, low sensitivity and narrow range of quantification) but also have some advantages (including ease of use, reduced risk of

contamination, reduced cost, and reliability even with samples stored at room temperature for extended periods of time [1, 32]). Although core Ag levels are thought to be related closely to HCV RNA titers, the correlation and linearity of core Ag levels have not yet been fully evaluated. In addition, these quantitative parameters are known to be affected by nucleotide and amino acid sequences at the target regions of the assays (5, 6, 28, 34), and this sequence variation depends on genotypes or predominant strains in specific geographical regions.

In this study, we established a Japanese reference panel of samples for evaluation of HCV RNA and core Ag levels by collecting donated blood specimens that tested positive for HCV RNA and anti-HCV antibodies. Using this reference panel, we evaluated the HCV loads in these specimens with two HCV RNA assay kits and five core Ag assay kits and assessed correlations among the data generated by these kits.

MATERIALS AND METHODS

Preparation of reference panel. To establish a reference panel for HCV quantitative assays, a total of 80 donated plasma samples were selected. All of these specimens, supplied by the Japanese Red Cross Blood Centers, tested positive for the presence of HCV RNA and anti-HCV antibodies. These samples, collected in Japan from May to September of 2007, were obtained from Japanese blood donor volunteers in various regions of

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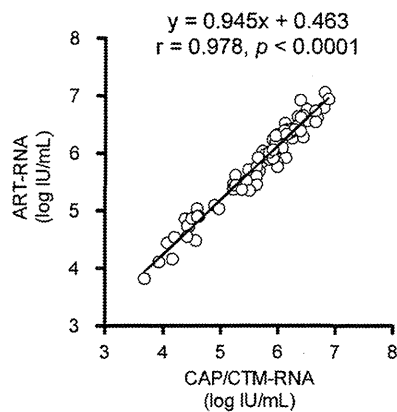


FIG 1 Correlation of HCV RNA titers as quantified by two commercial kits.

Japan. The samples were divided into 1-ml aliquots and stored at -80°C until use.

Quantification of HCV RNA and core Ag. The HCV RNA titer was measured with two real-time qRT-PCR kits, CAP/CTM-RNA (detection range, 1.5×10^1 to 6.9×10^7 IU/ml) and ART-RNA (detection range, 1.2×10^1 to 1.0×10^8 IU/ml). Additionally, samples were assessed using five HCV core Ag assay kits, including Architect-Ag (detection range, 3 to 20,000 fmol/liter), Lumipulse-Ag (detection range, 50 to 50,000 fmol/liter), Lumispot-Ag (detection range, 20 to 400,000 fmol/liter), ELISA-Ag (detection range, 44.4 to 3,600 fmol/liter), and IRMA-Ag (detection range, 20 to 20,000 fmol/liter). All assays were performed by the respective manufacturers at their research laboratories.

Sequencing and genotyping of HCV in reference panel samples. Viral RNA was extracted with the QIAamp viral RNA kit (Qiagen, Valencia, CA) from 140 μl of each plasma sample. HCV RNA was amplified by RT-PCR with primers corresponding to the 5' untranslated region (UTR) (43S-IH, 5'-CCTGTGAGGAACTACTGTCTTC-3'; c/s17-ssp, 5'-CCGG GAGACCATAGTGGTCTGCG-3') and the E1 region (1323R-IH, 5'-G CCGACCAGTTCATCATCAT-3'); the amplified products were sequenced directly. HCV genotypes of the isolated strains were assigned by phylogenetic analysis using an alignment with a representative strain of each genotype.

Statistical analysis. The correlations of obtained quantitative data were assessed by Pearson's correlation coefficient analysis, and values for r and P were calculated. A P value of <0.05 was considered to indicate statistical significance. Analysis was performed using Prism 5 software (GraphPad Software, Inc., La Jolla, CA).

Nucleotide sequence accession numbers. The accession numbers of C-01 to C-80 are AB705312 to AB705391, respectively.

RESULTS

Quantification of HCV RNA levels. The reference panel established in this work was used to measure HCV RNA levels with the CAP/CTM-RNA and ART-RNA kits. The correlation of the data obtained with the two kits is shown in Fig. 1. The RNA titers of these samples were distributed evenly, and all values were within the dynamic ranges of both assays. The HCV titers ranged from 3.68 to 6.88 and 3.82 to 7.08 log IU/ml in CAP/CTM-RNA and ART-RNA, respectively, and the correlation was significant ($r = 0.978$; $P < 0.0001$).

Quantification of HCV core Ag levels. HCV core Ag levels were measured using Architect-Ag, Lumipulse-Ag, Lumispot-Ag, ELISA-Ag, and IRMA-Ag kits. Among the 80 specimens in the reference panel, core Ag levels could be measured in all samples using Architect-Ag and ELISA-Ag kits, whereas core Ag levels

were below the detection limit in 4, 2, and 1 samples using Lumipulse-Ag, Lumispot-Ag, and IRMA-Ag kits, respectively (Fig. 2; also, see Fig. S1 in the supplemental material). Significant correlations were observed between assays of HCV core Ag and HCV RNA ($r = 0.9065$ to 0.9666 and $P < 0.0001$ compared with CAP/CTM-RNA data [Fig. 2]); $r = 0.8877$ to 0.9552 and $P < 0.0001$ compared with ART-RNA data [see Fig. S1 in the supplemental material]). The theoretical lower limits of detection of these assays were calculated by use of these correlation formulas and were 3.2 and 3.4 log IU/ml for Architect-Ag, 4.2 and 4.2 log IU/ml for Lumipulse-Ag, 3.7 and 3.9 log IU/ml for Lumispot-Ag, 3.6 and 3.8 log IU/ml for ELISA-Ag, and 3.6 and 3.8 log IU/ml for IRMA-Ag (compared to CAP/CTM-RNA and ART-RNA, respectively). These calculated detection limits were substantially higher than those for the RNA quantitative assays (1.18 and 1.08 log IU/ml for CAP/CTM-RNA and ART-RNA, respectively).

In addition, we found that several samples showed considerable deviation from the linear regression (Fig. 2; also, see Fig. S1 in the supplemental material). To identify the deviating samples, we used Bland-Altman plot analysis (Fig. 3; also, see Fig. S2 in the supplemental material). This plot shows the difference between the titer values of HCV RNA and core Ag as a function of the average of these two values. Several samples demonstrated discordance between the measured HCV RNA and core Ag levels. Among these samples, we focused on samples with discordant results in multiple core Ag assays compared to both RNA quantitative assays. For sample C-01, core Ag levels were underestimated when measured with Architect-Ag, Lumipulse-Ag, and Lumispot-Ag in comparison with CAP/CTM-RNA (Fig. 3) and when measured with Architect-Ag, Lumipulse-Ag, Lumispot-Ag, and IRMA-Ag in comparison with ART-RNA (see Fig. S2 in the supplemental material). Likewise, for sample C-73, core Ag levels were underestimated when measured with Architect-Ag, Lumipulse-Ag, and IRMA-Ag in comparison with CAP/CTM-RNA (Fig. 3) and when measured with Architect-Ag and Lumipulse-Ag in comparison with ART-RNA (see Fig. S2 in the supplemental material). Thus, sample-specific underestimation was observed in several HCV core Ag kits.

Nucleotide sequences in core region of reference panel samples. To clarify the sources of these underestimates of HCV core Ag levels, HCV RNA was extracted from each of the samples in the reference panel, and the nucleotide sequences of core regions were determined. Phylogenetic analysis with these sequences permitted classification of the individual strains by genotype. Of 80 samples in the reference panel, 1 (1.3%) was genotype 1a, 35 (43.8%) were genotype 1b, 26 (32.5%) were genotype 2a, and 18 (22.5%) were genotype 2b (Table 1; also, see Fig. S3 in the supplemental material). These strains were distributed evenly among reference strains of each genotype and cover the sequence diversity of strains isolated in Japan (see Fig. S3 in the supplemental material). The genotypes of samples associated with underestimated core Ag values (samples C-01 and C-73) were both classified as genotype 2a.

Predicted amino acid sequences of HCV core protein were aligned with the consensus core protein sequence for the genotype 1b strains obtained in this study (see Fig. S4 in the supplemental material). Excluding the genotype-specific sequence variations, a specific amino acid polymorphism was identified at amino acid (aa) residue 48 (Ala to Thr) in samples C-01 and C-73. Sample C-01, which yielded underestimated values in most core Ag assays, also possessed an additional polymorphism in the same region,

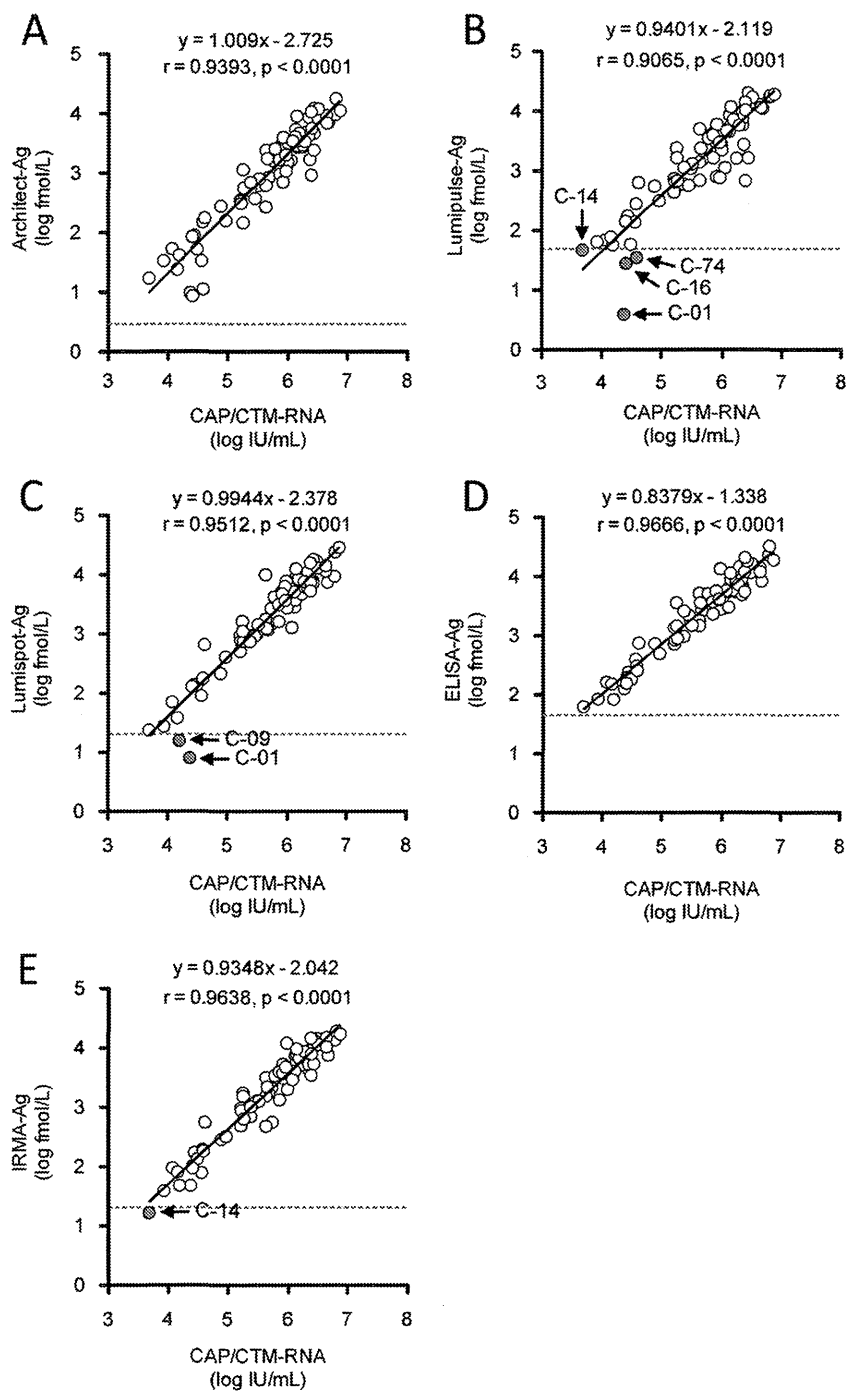


FIG 2 Correlation between CAP/CTM-RNA and core Ag levels as quantified by five commercial kits. Data for core Ag levels were converted to log fmol/liter prior to analysis. In each plot, the lower limit of detection of the respective core Ag assay is indicated by a dotted line. Data for samples below the lower detection limit of each assay are indicated by shaded circles labeled with the respective sample designations.

specifically an Arg-to-Gly substitution at aa 47. We suspected that these polymorphisms altered the antigenicity of the core protein, thereby reducing detected core Ag levels and leading to underestimation of values by the core Ag quantification kits. To assess the correlation of these polymorphisms with the underestimation of core Ag values, strains containing polymorphisms in this region (at aa 47 to 49 [Fig. 4]) were identified in Bland-Altman plots of HCV RNA and core Ag (Fig. 3; also, see Fig. S2 in the supplemental

material). A total of 12 strains exhibited polymorphisms at these positions, including 2 strains of genotype 1b, 8 of genotype 2a, and 2 of genotype 2b (Table 1). In the Bland-Altman plot of CAP/CTM-RNA and Architect-Ag, 4 of 12 values (for samples C-01, C-16, C-73, and C-74) were located under the line of the lower 95% limit of agreement (Fig. 3A). Likewise, in the plot of CAP/CTM-RNA and Lumipulse-Ag, 3 of 12 values (those for samples C-01, C-67, and C-73) were located under the line of the lower

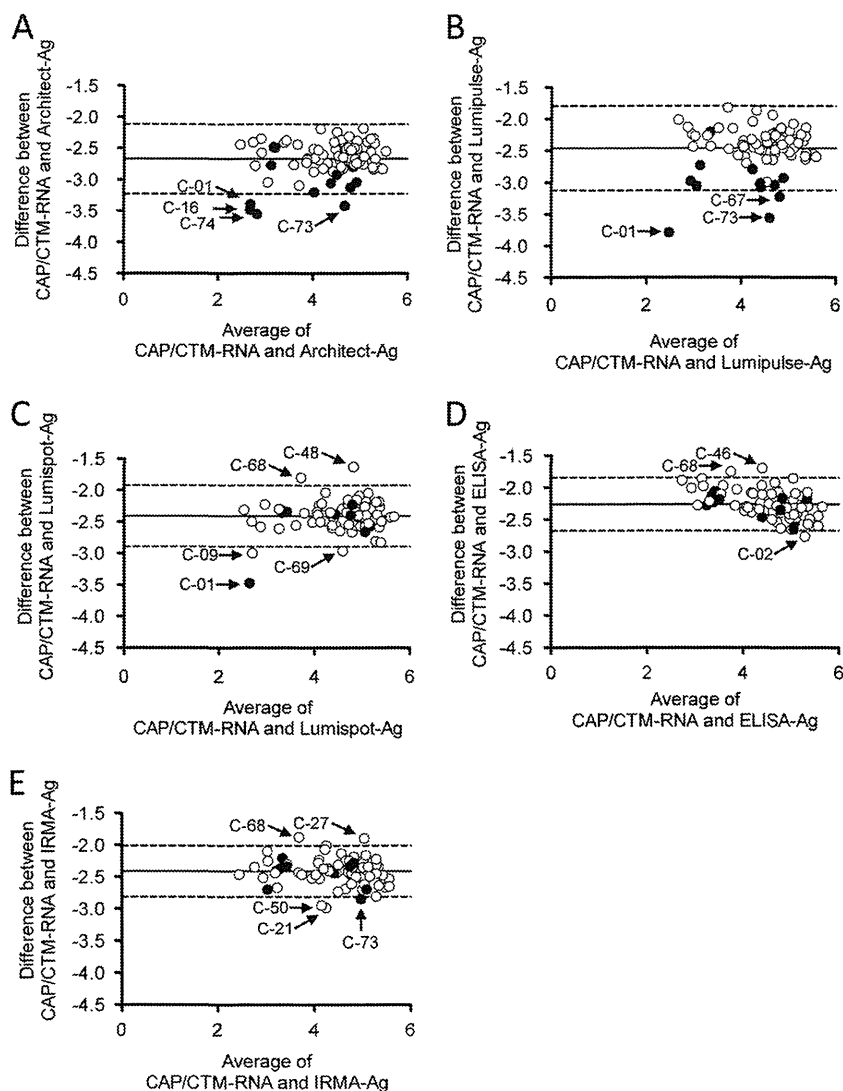


FIG 3 Bland-Altman plot analysis of CAP/CTM-RNA and core Ag levels as quantified by five commercial kits. These plots show the difference between the values of HCV RNA and core Ag as a function of the average of these two values. Data for core Ag levels were converted to log fmol/liter prior to analysis. The bias and 95% limits of agreements are indicated by solid and dashed lines, respectively. Data for samples with polymorphisms at amino acid residues 47 to 49 are indicated by solid circles. Data points outside the 95% limits are indicated by arrows labeled with the sample designations.

95% limit of agreement (Fig. 3B). In these plots, underestimation for samples that lacked these polymorphisms (at aa 47 to 49) was not detected. In the plot of CAP/CTM-RNA and Lumispot-Ag, only 1 sample (C-01) was located under the line of the lower 95% limit of agreement, but this sample exhibited the most discordant

value (Fig. 3C). In the plot of CAP/CTM-RNA and ELISA-Ag, no correlation between polymorphisms at these positions and underestimation was observed (Fig. 3D). In the plot of CAP/CTM-RNA and IRMA-Ag, sample C-73 was located under the line of the lower 95% limit of agreement, as were other samples that lacked polymorphisms at aa 47 to 49 (Fig. 3E). Similar trends were observed in comparison with ART-RNA levels (see Fig. S2 in the supplemental material). Based on these results, the levels of HCV core Ag measured with Architect-Ag and Lumipulse-Ag seem to be more strongly affected by single polymorphisms at these positions. In the case of Lumispot-Ag, underestimation may be limited to specimens with multiple polymorphisms at these positions.

TABLE 1 Number of reference panel strains with polymorphisms at amino acid residues 47 to 49 of the HCV core region

Genotype	No. (%) of strains	
	Total	With polymorphisms
1a	1	0
1b	35	2 (5.7)
2a	26	8 (30.8)
2b	18	2 (11.8)
Total	80	12 (15.0)

DISCUSSION

The quantification of HCV viral load is essential for selecting an appropriate antiviral strategy and for monitoring the efficacy of treatment. Since HCV is known to be highly variable and rapidly

aa	1	60
1b-cons.	MSTNPKPQRKTKRNTNRRPQDVKFFGGGQIVGGVYLLPRRGPRLGVRATRKTSERSQPRG	
C-16 (1b)P.....	
C-53 (1b)P.....	
C-01 (2a)GT.....	
C-03 (2a)L.....T.....	
C-12 (2a)	...T.....T.....	
C-65 (2a)	...T.....A.....	
C-67 (2a)T.....	
C-71 (2a)T.....	
C-73 (2a)T.....	
C-76 (2a)A.....	
C-08 (2b)P.....	
C-74 (2b)P.....	

FIG 4 Alignment of the first 60 amino acids of the HCV core region of strains with polymorphisms at amino acid residues 47 to 49. The position numbers are given at the top. Dots indicate identical amino acids. The consensus sequence of 1b strains (1b cons.) isolated in this study was determined and used as a reference sequence. Genotypes of strains are given in parentheses. Positions of polymorphisms are indicated by inverted triangles above the sequence alignment.

evolving (23, 26), the assays for quantifying this virus should be unaffected by sequence polymorphisms. In this study, we established a reference panel with HCV-positive samples and evaluated the correlation among multiple assays for HCV RNA and core Ag quantification.

Using this reference panel, we found that the results from two HCV RNA assay kits, CAP/CTM-RNA and ART-RNA, correlated with excellent agreement ($r = 0.978$, $P < 0.0001$ [Fig. 1]), although discrepancies for values generated by these two assays have been reported for strains of genotypes 1, 2, and 4 (5, 6, 34). In Japan, the prevalent genotypes are 1b, 2a, and 2b (11); no genotype 4 sample was included in our reference panel (Table 1). In quantification with CAP/CTM-RNA, underestimation of HCV RNA titer has been reported for French genotype 2 samples (5). In our panel, no underestimation was observed for data from genotype 2 samples compared to values obtained using ART-RNA. Therefore, underestimation in quantification with CAP/CTM-RNA is expected to be rare in Japanese samples, and the two assays for HCV RNA quantification should be considered accurate and reliable, at least for Japanese samples. Additionally, the prepared reference panel appears to be suitable for the evaluation of HCV quantification assays, because genotypes of samples in this panel are representative of those found in Japan and viral loads are distributed evenly across the range of expected titers.

The quantification of HCV core Ag is an alternative test for HCV infection and viral load. However, in this study, several core Ag quantitative assays failed to provide accurate results for all of the samples in the reference panel (Fig. 2). Some quantified values were below the kits' detection limits. This shortcoming was mainly attributable to the lower sensitivity of the core Ag assay kits; increased sensitivity is urged in the future development of HCV core Ag kits. Among the kits tested here, Architect-Ag assay exhibited the highest sensitivity and was sufficient for quantifying the viral load in all samples. However, even in the case of Architect-Ag, theoretical lower limits of detection, calculated by correlation formula using CAP/CTM-RNA and ART-RNA, were 3.2 and 3.4 log IU/ml, respectively; these detection limits still exceeded the lower limits of the HCV RNA quantification assays. Therefore, the sensitivity of the available HCV core Ag assays is still insufficient to detect low-titer HCV infections. Core Ag kits therefore may be unsuitable for the detection of breakthrough hepatitis during antiviral therapy or for the detection of HCV infection in a window period.

Comparison between HCV RNA and core Ag assays revealed good correlations, with r coefficients ranging from 0.8877 to 0.9666 and P values being less than 0.0001 (Fig. 2; also, see Fig. S1 in the supplemental material). Therefore, the HCV core Ag levels may serve as an alternative to HCV RNA levels when titers remain within the detection ranges of the core Ag kits. However, several discordances were detected when core Ag levels were compared with those of HCV RNA. For one sample in our panel (sample C-01), core Ag levels were lower than expected when quantified using any of the three core Ag kits (Architect-Ag, Lumipulse-Ag, and Lumispot-Ag) (Fig. 3; also, see Fig. S2 in the supplemental material). Another sample (C-73) also yielded lower-than-expected levels when assayed with Architect-Ag and Lumipulse-Ag kits. Sequence analysis of the core region revealed that polymorphisms at aa 47 and 48 correlated with these underestimates by core Ag kits (see Fig. S4 in the supplemental material). These results are consistent with our previous study, which suggested that core Ag levels of HCV strain JFH-1 were underestimated by the Lumipulse-Ag kit in comparison to the ELISA-Ag assay (28). Strain JFH-1 harbors an Ala-to-Thr substitution at aa 48; conversion of Thr to Ala at this position in JFH-1 was sufficient to overcome this underestimation. This region of the core Ag presumably corresponds to one of the epitopes recognized by the monoclonal antibodies used in the Lumipulse-Ag kit, such that polymorphisms at this position affected the antigenicity of the core protein. In this study, we found that the presence of other polymorphisms in this region (aa 47 to 49) correlated with reduced core Ag levels as detected by Lumipulse-Ag, as well as by other assays (Architect-Ag and Lumispot-Ag). Sample C-01 demonstrated a drastic deviation from expected core Ag levels in these assays (Fig. 3; also, see Fig. S2 in the supplemental material). The HCV strain in this sample contains two polymorphisms (Arg to Gly at aa 47 and Ala to Thr at aa 48); the multiple polymorphisms may impair antibody binding more severely and therefore result in underestimation of core Ag levels. Interestingly, this sample exhibited reasonable core Ag levels when assayed using ELISA-Ag. Thus, the underestimation of core protein levels in this sample was kit dependent, suggesting the targeting of distinct epitopes by the antibodies used in each of these kits. This hypothesis could not be confirmed, because the identity of the epitopes targeted by each kit is proprietary.

Of 12 samples with amino acid polymorphisms in this region, 2 (5.7%) were of genotype 1b, 8 (30.8%) were of genotype 2a, and

TABLE 2 Number of strains in the sequence database^a with polymorphisms at amino acid residues 47 to 49 of the HCV core region

Genotype	No. (%) of strains				
	Tested	With polymorphism			Total
		At aa 47 (R/C, G)	At aa 48 (A/T, P)	At aa 49 (T/A, P, L)	
1b	543	2 (0.36)	4 (0.74)	16 (2.96)	22 (4.1)
2a	24	0	6 (25.0)	1 (4.2)	7 (29.2)
2b	39	0	0	2 (6.9)	2 (6.9)

^a <http://s2as02.genes.nig.ac.jp/>.

2 (11.8%) were of genotype 2b (Table 1). Searches of the Hepatitis Virus Database (<http://s2as02.genes.nig.ac.jp/>) revealed that corresponding amino acid polymorphisms were observed in 22 of 543 strains (4.1%) of genotype 1b, 7 of 24 strains (29.2%) of genotype 2a, and 2 of 39 strains (6.9%) of genotype 2b (Table 2). These percentages were consistent with our observations in the proposed reference panel. These data (our results and those from the database) clearly indicate that genotype 2a strains are the most frequent source of underestimation of core Ag levels. Notably, our search of the sequence database did not yield any HCV strain with multiple polymorphisms in the region from aa 47 to 49, as we saw in our sample C-01. Therefore, strains with such multiple polymorphisms are rare so far, but detection of this isolate among donated blood specimens suggests that such HCV strains could be emerging in clinical samples. For patients harboring such strains, HCV viral load may be underestimated if measurement of HCV viral load is performed by core Ag assay. Such underestimates may result in erroneous selection of therapy, adversely affecting patient outcome. Thus, this shortcoming in HCV core Ag assay kits needs to be addressed.

There is a growing need for evaluation of clinical assay kits with domestic specimen reference panels, since the performance of these kits may be affected by the genotypes or polymorphisms of predominant strains in different geographic regions. To our knowledge, such an investigation of HCV clinical assay kits with domestic specimens has not previously been conducted in Japan. The Japanese HCV reference panel described here was generated with plasma samples collected from Japanese volunteers. Each sample was divided into small aliquots, and the panel was prepared in multiple sets. The samples in our HCV reference panel represent the predominant strains and genotypes seen in Japan. We expect that this reference panel will be of use for the development, evaluation, and optimization of HCV assay kits for the Japanese clinical market.

In conclusion, we have established a Japanese reference panel for evaluation of HCV quantification assays. Using this reference panel, we found that two assay kits for HCV RNA could quantify HCV titers concordantly. We also found that the data generated by HCV core Ag assay kits correlated with the results of HCV RNA assays. However, the nominal core Ag levels measured by several kits underestimated actual levels for HCV samples with polymorphisms at aa 47 to 49 of the core Ag. The panel established in this study is expected to be useful for estimating the accuracy of currently available and upcoming HCV assay kits; such quality control is essential for clinical usage of these kits.

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