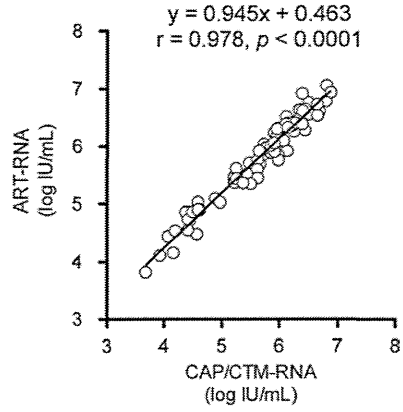


FIG 1 Correlation of HCV RNA titers as quantified by two commercial kits.

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, $1.5 \times 10^1$ to $6.9 \times 10^7$ IU/ml) and ART-RNA (detection range, $1.2 \times 10^1$ to $1.0 \times 10^8$ 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′-CCGGGAGAGCCATAGTGGTCTGCG-3′) and the E1 region (1323R-IH, 5′-GGCGACCAGTTCATCATCAT-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,

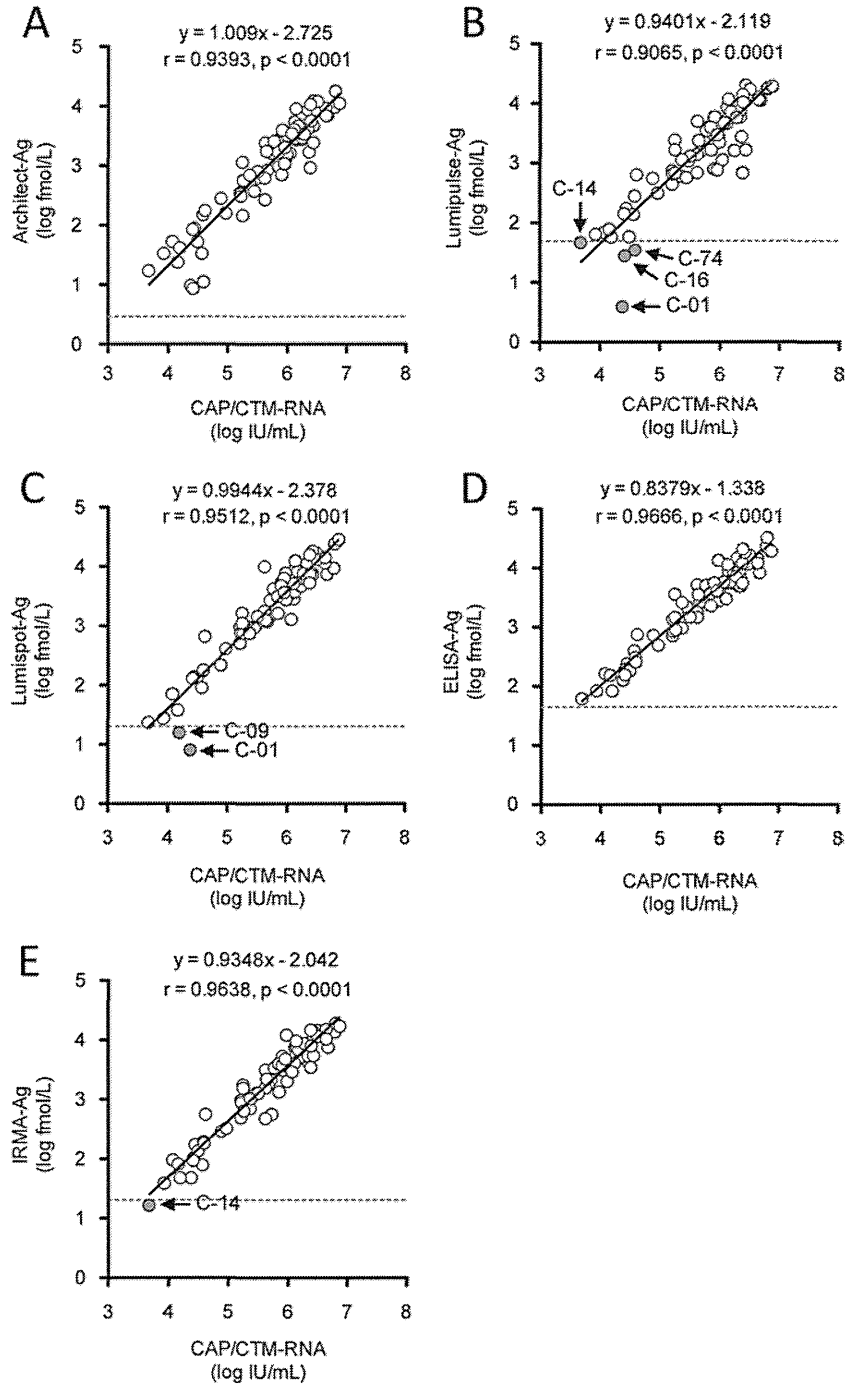


FIG 2 Correlation between CAP/CTM-RNA and core Ag levels as quantified by five commercial kits. Data for core Ag levels were converted to log fmol/liter prior to analysis. In each plot, the lower limit of detection of the respective core Ag assay is indicated by a dotted line. Data for samples below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

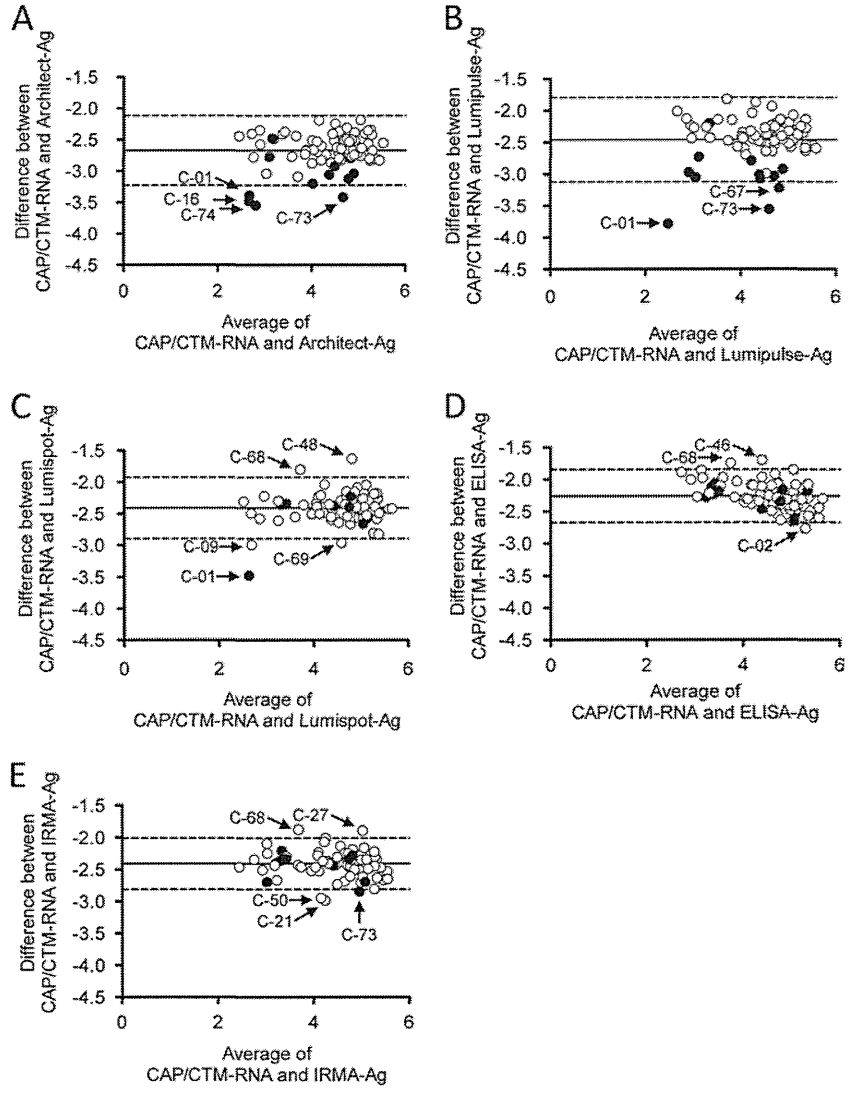


FIG 3 Bland-Altman plot analysis of CAP/CTM-RNA and core Ag levels as quantified by five commercial kits. These plots show the difference between the values of HCV RNA and core Ag as a function of the average of these two values. Data for core Ag levels were converted to log fmol/liter prior to analysis. 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

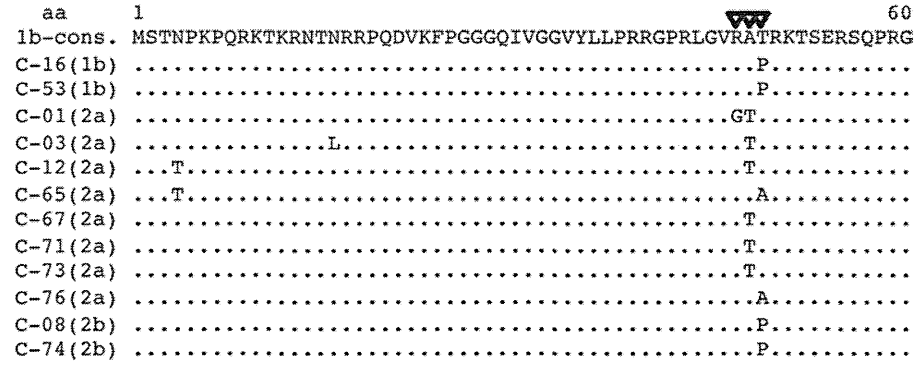


FIG 4 Alignment of the first 60 amino acids of the HCV core region of strains with polymorphisms at amino acid residues 47 to 49. The position numbers are given at the top. Dots indicate identical amino acids. The consensus sequence of 1b strains (1b cons.) isolated in this study was determined and used 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 | | | |
| | | At aa 47 (R/C, G) | At aa 48 (A/T, P) | At aa 49 (T/A, P, L) | Total |
| 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.

## ACKNOWLEDGMENTS

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## REFERENCES

1. **Alvarez M, Planelles D, Vila E, Montoro J, Franco E.** 2004. Prolonged hepatitis C virus seroconversion in a blood donor, detected by HCV antigen test in parallel with HCV RNA. Vox Sang. **86**:266–267.
2. **Aoyagi K, et al.** 1999. Development of a simple and highly sensitive enzyme immunoassay for hepatitis C virus core antigen. J. Clin. Microbiol. **37**:1802–1808.
3. **Aoyagi K, et al.** 2001. Performance of a conventional enzyme immunoassay for hepatitis C virus core antigen in the early phases of hepatitis C infection. Clin. Lab. **47**:119–127.
4. **Bossler A, et al.** 2011. Performance of the COBAS(R) AmpliPrep/COBAS TaqMan(R) automated system for hepatitis C virus (HCV) quantification in a multi-center comparison. J. Clin. Virol. **50**:100–103.
5. **Chevaliez S, Bouvier-Alias M, Brillet R, Pawlotsky JM.** 2007. Overestimation and underestimation of hepatitis C virus RNA levels in a widely used real-time polymerase chain reaction-based method. Hepatology **46**: 22–31.
6. **Elkady A, et al.** 2010. Performance of two real-time RT-PCR assays for quantitation of hepatitis C virus RNA: evaluation on HCV genotypes 1–4. J. Med. Virol. **82**:1878–1888.
7. **Enomoto M, et al.** 2005. Chemiluminescence enzyme immunoassay for monitoring hepatitis C virus core protein during interferon-alpha2b and ribavirin therapy in patients with genotype 1 and high viral loads. J. Med. Virol. **77**:77–82.
8. **Feld JJ, Hoofnagle JH.** 2005. Mechanism of action of interferon and ribavirin in treatment of hepatitis C. Nature **436**:967–972.
9. **Feld JJ, Liang TJ.** 2006. Hepatitis C—identifying patients with progressive liver injury. Hepatology **43**:S194–S206.
10. **Icardi G, et al.** 2001. Novel approach to reduce the hepatitis C virus (HCV) window period: clinical evaluation of a new enzyme-linked immunosorbent assay for HCV core antigen. J. Clin. Microbiol. **39**:3110–3114.
11. **Ikeda K, et al.** 1996. Hepatitis C virus subtype 3b infection in a hospital in Japan: epidemiological study. J. Gastroenterol. **31**:801–805.
12. **Kaiser T, et al.** 2008. Kinetics of hepatitis C viral RNA and HCV-antigen during dialysis sessions: evidence for differential viral load reduction on dialysis. J. Med. Virol. **80**:1195–1201.
13. **Leary TP, et al.** 2006. A chemiluminescent, magnetic particle-based immunoassay for the detection of hepatitis C virus core antigen in human serum or plasma. J. Med. Virol. **78**:1436–1440.
14. **Liang TJ.** 1998. Combination therapy for hepatitis C infection. N. Engl. J. Med. **339**:1549–1550.
15. **Liang TJ, Rehermann B, Seeff LB, Hoofnagle JH.** 2000. Pathogenesis, natural history, treatment, and prevention of hepatitis C. Ann. Intern. Med. **132**:296–305.
16. **Matsuura K, et al.** 2009. Abbott RealTime hepatitis C virus (HCV) and Roche Cobas AmpliPrep/Cobas TaqMan HCV assays for prediction of sustained virological response to pegylated interferon and ribavirin in chronic hepatitis C patients. J. Clin. Microbiol. **47**:385–389.

17. **Mederacke I, et al.** 2009. Performance and clinical utility of a novel fully automated quantitative HCV-core antigen assay. J. Clin. Virol. **46**:210–215.
18. **Medici MC, et al.** 2011. Hepatitis C virus core antigen: analytical performances, correlation with viremia and potential applications of a quantitative, automated immunoassay. J. Clin. Virol. **51**:264–269.
19. **Miedouge M, et al.** 2010. Analytical evaluation of HCV core antigen and interest for HCV screening in haemodialysis patients. J. Clin. Virol. **48**: 18–21.
20. **Morota K, et al.** 2009. A new sensitive and automated chemiluminescent microparticle immunoassay for quantitative determination of hepatitis C virus core antigen. J. Virol. Methods **157**:8–14.
21. **Moscato GA, et al.** 2011. Quantitative determination of hepatitis C core antigen in therapy monitoring for chronic hepatitis C. Intervirology **54**: 61–65.
22. **Nubling CM, Unger G, Chudy M, Raia S, Lower J.** 2002. Sensitivity of HCV core antigen and HCV RNA detection in the early infection phase. Transfusion **42**:1037–1045.
23. **Ogata N, Alter HJ, Miller RH, Purcell RH.** 1991. Nucleotide sequence and mutation rate of the H strain of hepatitis C virus. Proc. Natl. Acad. Sci. U. S. A. **88**:3392–3396.
24. **Park Y, Lee JH, Kim BS, Kim DY, Han KH, Kim HS.** 2010. New automated hepatitis C virus (HCV) core antigen assay as an alternative to real-time PCR for HCV RNA quantification. J. Clin. Microbiol. **48**:2253–2256.
25. **Pawlotsky JM.** 2006. Therapy of hepatitis C: from empiricism to eradication. Hepatology **43**:S207–S220.
26. **Robertson B, et al.** 1998. Classification, nomenclature, and database development for hepatitis C virus (HCV) and related viruses: proposals for standardization. Arch. Virol. **143**:2493–2503.
27. **Ross RS, et al.** 2010. Analytical performance characteristics and clinical utility of a novel assay for total hepatitis C virus core antigen quantification. J. Clin. Microbiol. **48**:1161–1168.
28. **Saeed M, et al.** 2009. Evaluation of hepatitis C virus core antigen assays in detecting recombinant viral antigens of various genotypes. J. Clin. Microbiol. **47**:4141–4143.
29. **Seeff LB, Hoofnagle JH.** 2002. National Institutes of Health Consensus Development Conference: management of hepatitis C: 2002. Hepatology **36**:S1–S2.
30. **Takahashi M, Saito H, Higashimoto M, Atsukawa K, Ishii H.** 2005. Benefit of hepatitis C virus core antigen assay in prediction of therapeutic response to interferon and ribavirin combination therapy. J. Clin. Microbiol. **43**:186–191.
31. **Tanaka E, et al.** 2000. Evaluation of a new enzyme immunoassay for hepatitis C virus (HCV) core antigen with clinical sensitivity approximating that of genomic amplification of HCV RNA. Hepatology **32**:388–393.
32. **Tanaka Y, et al.** 2003. High stability of enzyme immunoassay for hepatitis C virus core antigen-evaluation before and after incubation at room temperature. Hepatol. Res. **26**:261–267.
33. **Vermehren J, et al.** 2008. Differences between two real-time PCR-based hepatitis C virus (HCV) assays (RealTime HCV and Cobas AmpliPrep/Cobas TaqMan) and one signal amplification assay (Versant HCV RNA 3.0) for RNA detection and quantification. J. Clin. Microbiol. **46**:3880–3891.
34. **Vermehren J, et al.** 2011. Development of a second version of the Cobas AmpliPrep/Cobas TaqMan hepatitis C virus quantitative test with improved genotype inclusivity. J. Clin. Microbiol. **49**:3309–3315.
35. **Vermehren J, et al.** 2011. Multi-center evaluation of the Abbott RealTime HCV assay for monitoring patients undergoing antiviral therapy for chronic hepatitis C. J. Clin. Virol. **52**:133–137.

# HBV培養細胞系による新規抗ウイルス化合物のスクリーニング

渡 士 幸 一*

索引用語：HBV，抗ウイルス剤，スクリーニング，生活環，細胞株

## 1 はじめに

HBVを排除することを目的とした抗ウイルス剤としては現在主に，インターフェロン（IFN）あるいはペグIFNと，ラミブジン，エンテカビルをはじめとする逆転写酵素阻害剤に限られる．HIV/エイズ治療の例を挙げるまでもなく，感染症の薬物治療において有用な戦略のひとつとして，異なった標的をもつ抗ウイルス剤を複数利用することの重要性が挙げられる．抗ウイルス剤投与により早晩出現する薬剤耐性ウイルスは，複数の抗ウイルス剤を用いることにより，その出現頻度が低下することがよく知られている．

既存の抗HBV剤とは異なる新たな系統の抗ウイルス剤を同定するためには，培養細胞でHBV感染増殖を再現する実験系の樹立とそれを用いた化合物スクリーニングが有効な方法のひとつである．

本稿ではこのような試みの例を紹介させていただく．

## 2 HBV生活環について

まずHBVのウイルス生活環に関して概説する（図1）．HBVは標的肝細胞の表面への「吸着」ののち，「侵入」「脱殻」「核移行」を経てエピゾーマルな「closed covalently circular DNA（cccDNA）の形成」が行われる．cccDNAからは少なくとも4種類の長さの異なるウイルス性RNA（それぞれ約3.5 kb, 2.4 kb, 2.1 kb, 0.7 kb）が「転写」される．各RNAからはそれぞれcoreおよびpol（3.5 kb RNAより），large S（2.5 kb RNAより），middle SおよびsmallS（2.1kb RNAより），x（0.8 kb RNAより）の各ウイルス性蛋白質が「翻訳」される．xは転写調節作用をもち，cccDNAからのHBV RNA転写を活性化する．large S, middle SおよびsmallSはウイルスのエンベロープ蛋白質である．細胞質でcoreが多量体化したキャプシドとpolは，3.5 kb RNA（プ

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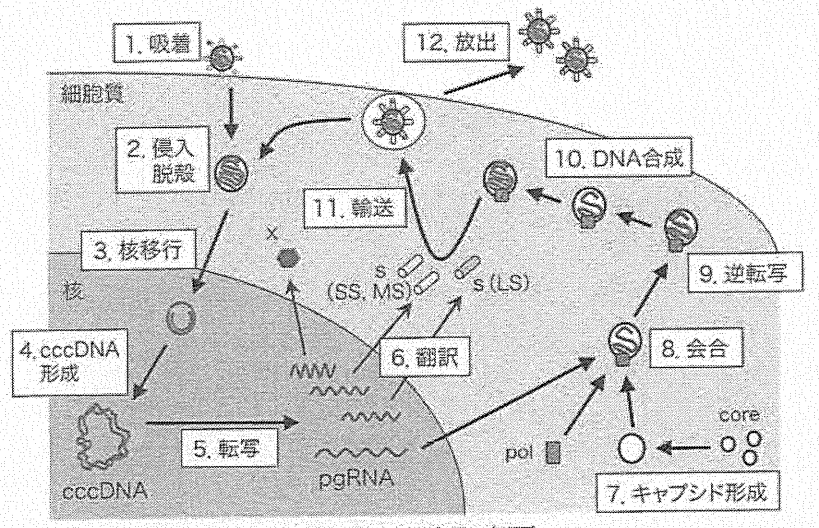



図1　HBV生活環の概要

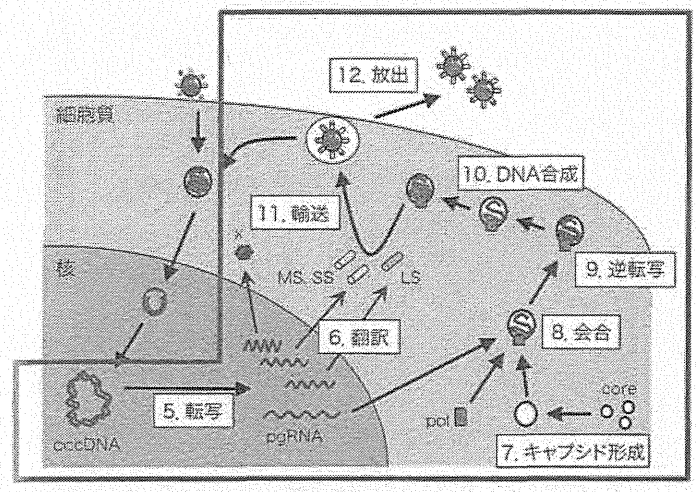
HBVは標的細胞への「吸着」の後，「侵入」「脱殻」を経て「核移行」する．核内で「cccDNA形成」し，ここから少なくとも4種類の長さの異なるHBV RNAを「転写」する．HBV RNAからウイルス性蛋白質x，s [small s (SS)，middle s (MS)，large s (LS)]，pol，coreが「翻訳」される．coreは多量体化することにより「キャプシド形成」し，さらにpolやpregenomic RNA (pgRNA)とともに「会合」する．core粒子内でpolがpgRNAを「逆転写」してcDNAを産生し，さらにpgRNAが消化された後これを鋳型として「DNA合成」により相補鎖cDNAが合成される．core粒子は細胞内「輸送」の過程でsを獲得し，細胞外へと「放出」される．またcore粒子の一部は再び核内へ移行し，この生活環の持続的維持に再利用される．

レゲノムRNAとも呼ばれる）と「会合」し，core粒子を形成する．core粒子内でpolによりプレゲノムRNAがDNAに「逆転写」され，続いてプレゲノムRNAが消化，さらにDNAを鋳型にして相補鎖「DNA合成」が行われる．core particleは細胞膜へ「輸送」される過程でlarge S，middle SおよびsmallSといったエンベロープを獲得し，細胞外へと「放出」される．またcore particleの一部は再び核内へ移行し，cccDNA形成のため「再利用」される．このようにウイルス生活環のおおまかな流れは判明してはいるものの，これらがどのような細胞性因子を利用して，どのようなしくみで行われているのか，については不明な点が多い．

## 3　HBV感染複製を評価できる実験系

HCV研究においては，JFH1株およびこれに由来するキメラ株と，ヒト肝癌由来Huh-7細胞という「ウイルス－宿主細胞」の組み合わせを主に用いることにより，細胞への感染～複製増殖～放出とそれに続く新たな細胞への感染という，持続的なウイルス増殖サイクルが培養細胞内で再現できる．しかしHBVではこのような持続的感染増殖系はいまだ存在しない．そこでウイルス生活環の分子メカニズムを研究するには，生活環の少なくとも一部のみを評価できる実験系を用いて解析を行う．

HBV生活環の転写以降のステップは，約




1) 一過性発現系
HBVをコードするプラスミドをHuh-7細胞, HepG2細胞などの肝細胞株にトランスフェクションする.

2) 恒常的発現細胞株
HBVプラスミドが細胞ゲノムにインテグレーションされて, 恒常的にHBVを産生する細胞株. Hep2.2.15細胞など.

3) 誘導発現細胞株
HBV発現をテトラサイクリンなどで誘導できるプラスミドが細胞ゲノムにインテグレーションされた細胞株. HepAD38細胞など.

図2　HBV生活環後期過程を評価できる実験系
HBV生活環のうち転写以降を再現する培養細胞実験系としては, 主に一過性発現系, 恒常的発現細胞株, 誘導発現細胞株がある.

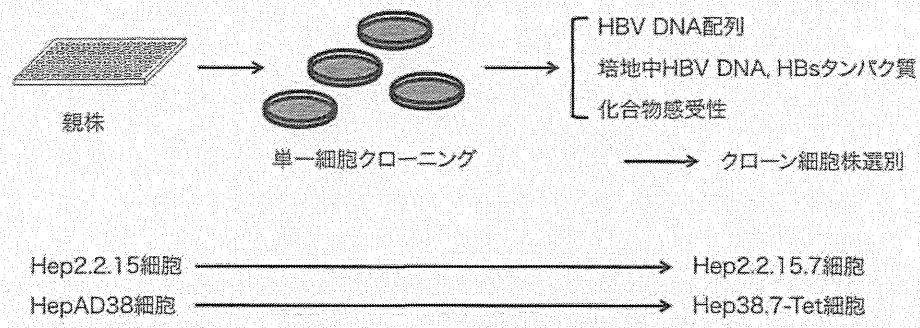



図3　Hep2.2.15細胞, HepAD38細胞からの単一細胞クローニング
Hep2.2.15細胞, HepAD38細胞から単一細胞クローニングにより, Hep2.2.15.7細胞およびHep38.7-Tet細胞を得た. これらの細胞は親株に比較してHBV DNA産生が高く, ラミブジン感受性が高いため, 化合物のHBV複製への効果を解析するのに適していると考えられる.

1.2倍長以上のHBV DNAを肝細胞由来細胞株にトランスフェクションすることで解析できる(図2). この過程は上記HBV DNAをコードするプラスミドを一過性にトランスフェクションすることにより, あるいはこのようなプラスミドが細胞内ゲノムに安定的に組み込まれた恒常的発現細胞株を用いても再現できる. 後者に関しては例えば1986年にDr. Acsのグループにより樹立されたHep2.2.15細胞が多くの研究グループにより用いられている[1]. またcoreプロモーターの一部をテトラサイクリン制御性プロモーターに置換することにより, テトラサイクリン存在/非存在でHBV産生の有無を制御できる誘導型発現細胞系を樹立することもでき, 例えばDr. Seegerらにより報告されたHepAD38細胞[2]やDr. Guoらにより樹立されたHepDE19細胞[3]などがある. これらの実験系ではHBV DNAからの転写から始まり, 会合, 逆転写などを経てウイルス放出(便宜的にHBV生活

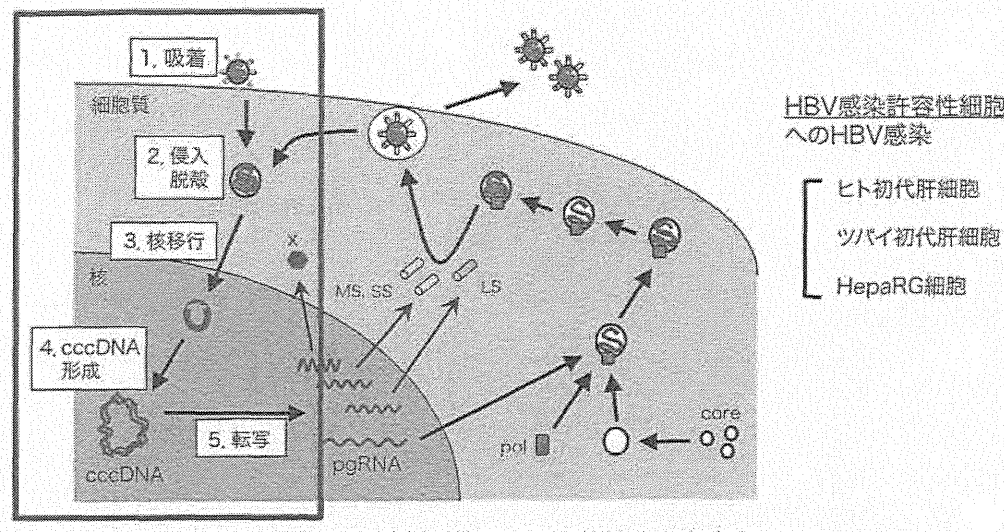



図4　HBV生活環前期過程を評価できる実験系

HBV生活環のうち吸着からはじまる前期過程は，HBV感染許容性細胞にHBVを感染させることで再現できる．感染許容性細胞としては，ヒト初代肝細胞，ツパイ初代肝細胞およびHepaRG細胞などが知られる．

環後期過程と呼ぶ)までが持続的に行われているため，後期過程の分子メカニズムの解析やこの過程を標的とした抗ウイルス剤のスクリーニングに有効である．

私たちのグループでは，Hep2.2.15細胞およびHepAD38細胞から単一細胞クローンを選別した(図3)．これらを用いた解析ではHBV DNAやHBs産生，また逆転写酵素阻害剤感受性が細胞クローンごとに異なっており，それぞれの研究目的に応じたクローンを選択することにより，より効率的な解析が可能であると考えられる．

HBV転写以前を含む生活環前期過程を評価するには，これらとは異なる実験系を用いる必要がある．HBs蛋白質からなるsubviral particleを用いて標的細胞への侵入を解析することもできるが[4]，一方でより本来の感染を模倣した系として，感染性HBV粒子を感染許容性細胞に感染させて，吸着，侵入およびそれ以降の生活環ステップを解析することができる(図4)．HBV感染許容性細胞としてはこれまでにヒト初代肝細胞[5,6]，ツパイ初代肝細胞[7]などが知られている．培養細胞株の多くはHBVに対して明らかな感染許容性は示さないが，2002年にヒト肝細胞由来HepaRG細胞がHBV感染をサポートすることが報告された[8]．HepaRG細胞は分化能を有した細胞株であり，DMSO，ヒドロコルチゾンの持続的処理によりヒト初代肝細胞に近い形質を獲得し，HBV感染許容性となる．この細胞を用いたHBV吸着侵入の解析は複数の研究グループから報告されており[9,10]，現在HBV感染実験には最も汎用される細胞株である．この系を用いることにより，吸着，侵入などの前期過程を含んだHBV生活環を再現よく解析することができる．

## 4　HBV後期過程に作用する抗HBV化合物のスクリーニング

これまでHBV複製増殖を直接阻害する抗HBV剤としては逆転写酵素阻害剤のみである．そこで生じる疑問が「逆転写ステップ以

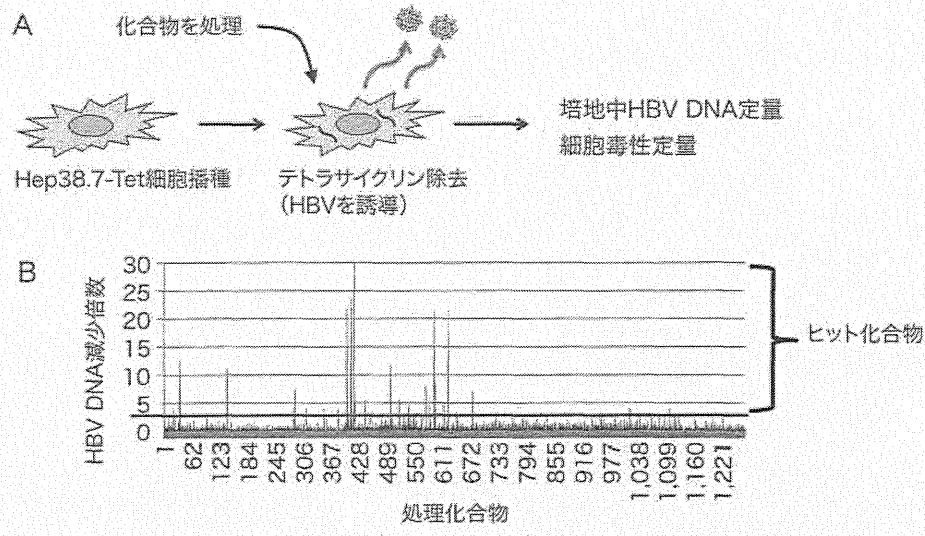



図5　Hep38.7-Tet細胞を用いた化合物スクリーニング

A：スクリーニングプロトコールの1例．Hep38.7-Tet細胞を播種した後にテトラサイクリン除去によりHBV発現を誘導する．同時に化合物を処理し，培地中HBV DNAを定量にすることでウイルス粒子産生への効果を調べる．また細胞毒性をあわせて検討する．

B：同様のスクリーニングをHepAD38細胞で行った結果の1例．相対HBV DNA量を1/3以下に下げたものをヒット化合物とした．

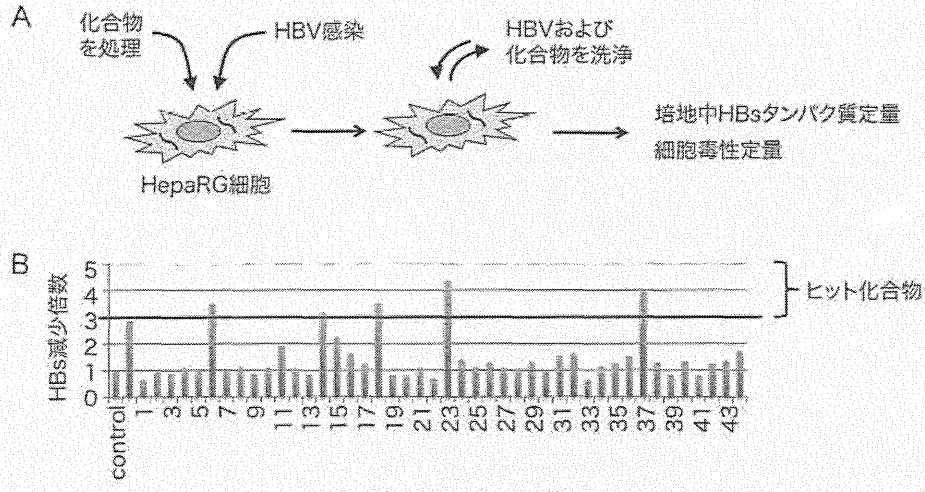



図6　HepaRG細胞を用いた化合物スクリーニング

A：スクリーニングプロトコールの1例．HepaRG細胞に化合物を処理するとともにHBVを感染させる．HBVおよび化合物を洗浄後，培地中HBs蛋白質を定量にすることでHBV感染への効果を調べる．また細胞毒性をあわせて検討する．

B：スクリーニング結果の1例．相対HBs蛋白質量を1/3以下に下げたものをヒット化合物とした．

外を阻害する抗HBV化合物は存在し得るのかどうか」「もしそのようなものがあるとすればどのステップが創薬の標的となり得るか」という点である．もしこのような化合物が存在することが示されれば，今後逆転写ステップ以外を標的とする化合物を探索することが新規抗HBV剤同定に有効であるといえる．私たちの研究グループではまずHepAD38細胞を用いた培養細胞ベース系を用い，このような非逆転写阻害抗HBV化合物のスクリーニングを行った(図5)．1,000化合物強のライブラリーを用いたスクリーニングの結果，培地に放出されたHBV DNA量を1/3以下に低下させるものとして38化合物，このうち1/10以下に強く低下させるものが8化合物であった．続いてこれらの化合物の標的ステップを解析したところ，キャプシド形成，ウイルス会合，DNA合成などのさまざまなステップを阻害するものが含まれていることが示唆された．

## 5 HBV感染実験系を用いた抗HBV化合物のスクリーニング

一方，HBV生活環前期過程を阻害する化合物のスクリーニングはHepaRG細胞を用いたHBV感染実験系により行った(図6)．HBV感染後の細胞から放出されるHBs蛋白質をELISA法により定量した．その結果，1/3以下にHBs蛋白質産生を低下させる化合物が得られたが，その後の解析によりこれらのうち少なくとも3化合物は吸着ではなく，侵入を阻害する可能性が考えられた．

## 6 まとめ

このように化合物スクリーニングにより侵入，キャプシド形成，ウイルス会合，DNA合成のさまざまなステップを阻害する化合物が複数得られた．以上の結果は逆転写以外のステップを阻害する化合物が存在し，それらは培養細胞ベース系の化合物スクリーニングにより得られることを示している．今後さらに多くの化合物をスクリーニングすること，あるいは得られた化合物の誘導体解析を行うことにより，さまざまな標的を阻害する抗HBV剤が得られることが期待される．

## 7 おわりに

以上，培養細胞ベースの抗HBV化合物スクリーニングの例を紹介した．今回紹介した実施例のような実験系では，最初から標的ステップを制限することなくスクリーニングを行うことが特徴であり，ヒット化合物の抗HBVメカニズムを解析することにより，標的ステップおよび標的分子を同定することができる．このような解析においては，新規抗ウイルス剤の同定だけではなく，新たなウイルス生活環の分子機構を明らかにできる可能性がある．

一方，最初から標的ステップを絞ってスクリーニングすることもできる．HBV生活環の複数のステップを評価できる実験系のかわりに，例えば転写，キャプシド形成，逆転写などのステップのみを再現する培養細胞ベースあるいは試験管内実験系を用いてスクリーニングする方法である．このような方法の利点としては，ヒット化合物を得られた時点で，少なくとも化合物の標的ステップが明らかになっており，その後の展開を短期間で行うことができる点があげられる．一方試験管内スクリーニングの欠点としては，ヒット化合物の細胞透過性および細胞毒性を当初のアッセイでは全く評価していないため，実際に細胞でアッセイを行うとHBV複製増殖を阻害できない，あるいは細胞毒性が高く抗HBV剤

として適さないなどの問題が生じ得る点である．

以上のような利点と欠点をかんがみるに，新規抗HBV剤を同定するには，培養細胞ベースおよび試験管内系を組みあわせて，多方面のアッセイから化合物スクリーニングを行うことが重要であると考えられる．これらの目的には，もちろん新たなHBV複製増殖系の開発およびスクリーニング系の開発が重要であることはいうまでもない．HBV基礎研究の推進によって，今後このような研究も大きく進展すると期待される．

## 文　献


1) Sells MA, Chen ML, Acs G : Production of hepatitis B virus particles in Hep G2 cells transfected with cloned hepatitis B virus DNA. Proc Natl Acad Sci USA 84 : 1005–1009, 1987
2) Ladner SK, Otto MJ, Barker CS et al : Inducible expression of human hepatitis B virus (HBV) in stably transfected hepatoblastoma cells: a novel system for screening potential inhibitors of HBV replication. Antimicrob Agents Chemother 41 : 1715–1720, 1997
3) Guo H, Jiang D, Zhou T et al : Characterization of the intracellular deproteinized relaxed circular DNA of hepatitis B virus: an intermediate of covalently closed circular DNA formation. J Virol 81 : 12474–12484, 2007
4) Hao X, Shang X, Wu J et al : Single-particle tracking of hepatitis B virus-like vesicle entry into cells. Small 7 : 1212–1218, 2011
5) Gripon P, Diot C, Thézé N et al : Hepatitis B virus infection of adult human hepatocytes cultured in the presence of dimethyl sulfoxide. J Virol 62 : 4136–4143, 1988
6) Ochiya T, Tsurimoto T, Ueda K et al : An in vitro system for infection with hepatitis B virus that uses primary human fetal hepatocytes. Proc Natl Acad Sci USA 86 : 1875–1879, 1989
7) Walter E, Keist R, Niederöst B et al : Hepatitis B virus infection of tupaia hepatocytes in vitro and in vivo. Hepatology 24 : 1–5, 1996
8) Gripon P, Rumin S, Urban S et al : Infection of a human hepatoma cell line by hepatitis B virus. Proc Natl Acad Sci USA 99 : 15655–15660, 2002
9) Engelke M, Mills K, Seitz S et al : Characterization of a hepatitis B and hepatitis delta virus receptor binding site. Hepatology 43 : 750–760, 2006
10) Macovei A, Radulescu C, Lazar C et al : Hepatitis B virus requires intact caveolin-1 function for productive infection in HepaRG cells. J Virol 84 : 243–253, 2010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