

Necessity for Reassessment of Patients with Serogroup 2 Hepatitis C Virus (HCV) and Undetectable Serum HCV RNA

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We encountered a patient positive for anti-hepatitis C virus (HCV) whose serum HCV RNA was undetectable with the Roche AmpliPrep/Cobas TaqMan HCV assay (CAP/CTM) version 1 but showed a high viral load with the Abbott RealTime HCV assay (ART). Discrepancies in the detectability of serum HCV RNA were investigated among 891 consecutive patients who were positive for anti-HCV. Specific nucleotide variations causing the undetectability of HCV RNA were determined and confirmed by synthesizing RNA coding those variations. Serum samples with the discrepancies were also reassessed by CAP/CTM version 2. Among the 891 anti-HCV-positive patients, 4 patients had serum HCV RNA levels that were undetectable by CAP/CTM version 1 despite having levels of >5 log IU/ml that were detected by ART. All four patients had HCV genotype 2a and high titers of anti-HCV. Sequencing of the HCV 5' noncoding regions revealed 2 common variations, A at nucleotide (nt) 145 and T at nt 151. Synthesized RNAs of the HCV 5' noncoding region with standard (NCR145G151C) and variant nucleotides at nt 145 and nt 151 were quantified with CAP/CTM. RNAs of NCR145G151C and NCR145G151T were quantifiable with CAP/CTM version 1, while those of NCR145A151T and NCR145A151C went undetected. The substitution from G to A at nt 145 specifically conferred this undetectability, while this undetectability was reverted in synthesized HCV RNA with correction of this variation. Reassessment of these samples by CAP/CTM version 2 resulted in similar levels of HCV RNA being detected by ART. We conclude that HCV patients with undetectable HCV RNA by CAP/CTM version 1 should be reassessed for viral quantification.

Hepatitis C virus (HCV) infection causes chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma (1, 2). More than 170 million people worldwide are infected with HCV, creating a serious global health problem. Monitoring of serum HCV RNA levels during antiviral therapy is essential for the management of HCV infection (3). Sustained virological response is generally evaluated according to whether HCV RNA can be detected in the serum 12 or 24 weeks after the cessation of treatment. Recent monitoring of serum HCV RNA has been done mostly by real-time PCR methods, because real-time PCR methods are sensitive, with low limits of detection, and have broad dynamic ranges of quantification (4, 5).

The Roche AmpliPrep/Cobas TaqMan HCV assay (CAP/CTM) (Roche Molecular Systems, Pleasanton, CA) version 1 may underestimate or overestimate HCV RNA levels in a number of patients infected with HCV genotypes 2 and 4 because of mismatch of the primers or probes with the viral sequences (6). The undetectability due to sequence mismatch in CAP/CTM version 1 has been overcome for HCV genotype 4 by CAP/CTM version 2 (7). The Abbott RealTime HCV assay (ART) (Abbott Molecular, Des Plaines, IL) and CAP/CTM version 2 have also been reported to have sensitivities and accuracies superior to those of CAP/CTM version 1.

The present study is the first reported investigation of patients with HCV genotype 2a whose serum HCV RNA was undetectable with CAP/CTM version 1 despite a high viral load detected by ART. We clarified the cause of the undetectability of HCV and estimated the prevalence of this discrepancy among patients with positive results from the anti-HCV test. The serum samples with discrepancies were also reassessed by CAP/CTM version 2, resulting in similar levels of HCV RNA determined by ART.

MATERIALS AND METHODS

Patients. The present study enrolled consecutive patients who had positive results on the anti-HCV test (Lumipulse Presto Ortho HCV; Fujirebio, Tokyo, Japan) and were admitted to the gastrointestinal unit of Okayama University Hospital between 2008 and 2012 for further examination or therapy for liver cirrhosis, esophageal and gastric varices, or hepatocellular carcinoma. Liver histologies were evaluated according to the criteria of Desmet et al. (8). HCV serogroups were assessed by the HCV serogrouping assay (HCV Gr; Sysmex International Reagents, Kobe, Japan), which can subgroup the patients in HCV serogroups 1 and 2, corresponding to HCV genotypes 1 and 2, respectively, with HCV group-specific anti-nonstructural region 4 antibodies. This assay is available not only for patients with chronic HCV infection, but also for those with resolved HCV. This study was performed in accordance with the Helsinki Declaration, and the protocol was approved by the ethics committee of the institute. This study was registered with the University Hospital Medical Information Network Clinical Trials Registry (UMIN 000001031). All patients provided informed consent before enrollment in the study.

Quantification of HCV RNA. Serum HCV RNA quantification was performed by the reverse transcription-quantitative PCR (RT-qPCR) system of CAP/CTM version 1 with automated sample preparation on a Cobas AmpliPrep extractor from 850 μ l of serum, and the Cobas TaqMan 48 analyzer was used for automated real-time PCR amplification and detection of PCR products, according to the manufactur-

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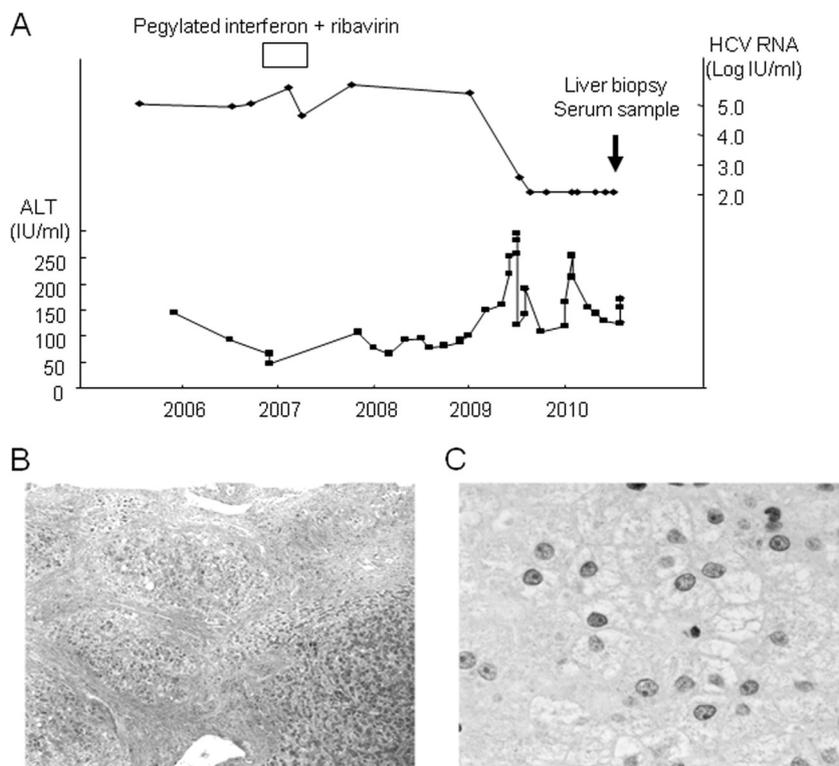


FIG 1 Clinical course and histology of patient 1. (A) Changes in serum HCV RNA and alanine aminotransferase. (B) Liver histology indicating liver cirrhosis (AZAN staining, 4-fold original magnification). (C) Immunohistochemical staining with a monoclonal antibody specific for HCV core demonstrating granular signal in the hepatocytes (400-fold original magnification).

er's instructions. When the HCV RNA was undetectable, additional quantification of HCV RNA was done within a month by ART with serum samples stored at -80°C . The results of HCV RNA were also compared with the levels of HCV core antigens (Architect HCV Ag; Abbott, Tokyo, Japan) and the reassessed results of HCV RNA by CAP/CTM version 2.

Analysis of sequence in the HCV 5' noncoding region. Serum RNA was extracted by means of a QIAamp viral RNA minikit according to the manufacturer's protocol (Qiagen, Tokyo, Japan). HCV RNA was amplified by RT-PCR with primers corresponding to the HCV 5' noncoding region for HCV genotype 2a as modified from a previous report (6). The primers were 5'NCRS (5'-GGGCGACACTCCGCCATGAA-3', nucleotide [nt] 17 to 36) and 5'NCRAS (5'-CCCTGCGCGGCAACAAGTA-3', nt 462 to 444). The first and second PCR rounds included an initial denaturation step at 95°C for 1 min, followed by 30 cycles of denaturation at 95°C for 30 s, annealing at 52°C for 30 s, and elongation at 70°C for 30 s, followed by a final elongation step at 70°C for 5 min. Direct sequencing was carried out by BigDye termination cycle sequencing using an ABI Prism 310 genetic analyzer (Applied Biosystems, Foster City, CA).

RNA synthesis of the HCV 5' noncoding region. Two vectors expressing the HCV 5' noncoding region between nt 17 and 462 were constructed, and their sequences were identical except for G at nt 145 and C at nt 151 for NCR145G151C (the detectable standard), A at nt 145 and T at nt 151 for NCR145A151T, G at nt 145 and T at nt 151 for NCR145G151T, and A at nt 145 and C at nt 151 for NCR145A151C. NCR145A151T was the undetectable variant obtained from the serum of patient 1. NCR145G151C, NCR145G151T, and NCR145A151C were constructed from NCR145A151T by PCR mutagenesis with primers containing base alterations. The PCR amplicons were cloned into pCR2.1 by means of a TA cloning kit according to the manufacturer's protocol (Life Technologies, Tokyo, Japan). The sequences of these inserts were confirmed by BigDye termination cycle sequencing (Applied Biosystems). The plasmids

were utilized for RNA synthesis by means of a MEGAscript kit according to the manufacturer's protocol (Life Technologies). Synthesized RNA was quantified with automated sample preparation from $850\ \mu\text{l}$ of the RNA sample by CAP/CTM version 1 and $500\ \mu\text{l}$ of the RNA sample by CAP/CTM version 2, respectively, on the Cobas TaqMan instruments, according to the manufacturer's instructions.

Immunohistochemistry of HCV core. The presence of HCV core protein was assessed by immunohistochemistry. The liver tissues of the patients were fixed in 10% buffered formalin and embedded in paraffin. The sections were treated with citrate buffer (10 mM citric acid and 0.05% Tween 20 [pH 6.0]) for 12 min at room temperature for antigen retrieval. The sections underwent a reaction with a monoclonal antibody for HCV core protein (CP-9; Institute of Immunology, Tokyo, Japan) after non-specific binding was blocked with casein. The intrinsic peroxidase activity was blocked by immersing sections in a methanol solution containing 0.3% hydrogen peroxide. The Envision detection system was applied with 2,4-diaminobutyric acid (DAB) (Dako, CA). Two negative controls were applied, one without the first monoclonal antibody and the other from the HCV-seronegative patients.

RESULTS

Our first patient in whom serum HCV RNA was undetectable with CAP/CTM version 1 despite a high viral load with ART. A 49-year-old male patient (patient 1) suffered from chronic hepatitis due to HCV genotype 2a, with an abnormal level of alanine aminotransferase of $>100\ \text{IU/ml}$ (Fig. 1A). He received antiviral therapy of pegylated interferon and ribavirin. His therapeutic response was only partial, and his liver function test results remained abnormal thereafter. Two years later, an abrupt decrease of serum HCV RNA was observed, and for 2 years, at every 3-month quantification with CAP/CTM version 1, no serum HCV

TABLE 1 Characteristics of patients with serum HCV RNA levels that were undetectable by CAP/CTM^a

Patient characteristic	Patient 1	Patient 2	Patient 3	Patient 4
Age (yr)	49	54	54	56
Gender	Male	Male	Male	Male
Liver histology (fibrosis/activity) ^d	4/3	4/2	4/1	Not done
Alanine aminotransferase (IU/liter)	124	29	62	51
Platelet count (per mm ³)	89,000	55,000	134,000	110,000
Anti-HCV antibody (COI) ^b	66.3	55.8	71.8	53.6
HCV RNA with ART ^c (log IU/ml)	5.9	5.6	5.9	5.6
HCV core antigen (fmol/liter)	1,340	3,320	623	1,586
HCV genotype	2a	2a	2a	2a

^a CAP/CTM, Roche AmpliPrep/Cobas TaqMan HCV assay.^b HCV, hepatitis C virus; COI, cutoff index.^c ART, Abbott RealTime HCV assay.^d Liver histology according to the criteria of Desmet et al. (8).

RNA was detected, although his level of alanine aminotransferase remained abnormal. Liver biopsy was performed for further examination for abnormal liver function and revealed that his liver disease had advanced to liver cirrhosis with interface hepatitis (Fig. 1B). The existence of HCV core antigens was confirmed in both the serum (Table 1) and the liver (Fig. 1C). Furthermore, reassessment of HCV RNA with ART showed a high viral load in his serum.

Characteristics of patients with HCV RNA detectable by ART but not by CAP/CTM. Among the 891 consecutive patients with positive results on the anti-HCV test who were admitted to the gastrointestinal unit for liver-related disorders due to HCV, CAP/CTM version 1 detected no HCV RNA in 69 patients; of these, 38 patients were sustained viral responders to previous interferon therapy, and 31 patients had not undergone antiviral therapies. Three patients who had not undergone antiviral therapies, and patient 1, who had undergone antiviral therapy, had serum HCV RNA levels of >5 log IU/ml by HCV RNA quantification with ART. The characteristics of these four patients are summarized in Table 1. All four patients were males in their forties or fifties. Three of these patients (not including patient 1) had not undergone antiviral therapies. Three (including patient 1) had histological liver cirrhosis. All four of these patients commonly had high titers of anti-HCV antibody and HCV genotype 2a. Coinfection with hepatitis B virus or human immunodeficiency virus was ruled out. Even though CAP/CTM version 1 produced false-negative results, we doubted that some of the 38 patients who were sustained viral responders might still have HCV, and serum HCV RNA was not detected by ART in these sustained viral responders.

Comparison of sequences of the 5' noncoding region of HCV. We hypothesized that HCV RNA that was undetectable with CAP/CTM version 1 might occur due to the mismatch of the primers or probes with the sequences of the target regions. Therefore, the sequences of the HCV 5' noncoding regions were determined by direct sequencing for all 4 patients whose HCV RNA was undetectable in the serum with CAP/CTM version 1, and their sequences were compared with the sequences of HCV genotypes 1a, 1b, 2a, 2b, and 3a using H77 as a reference (Fig. 2). The sequences of these 4 patients commonly showed genotype 2a-specific features in the nt 218 to 224 and nt 240 to 243 ranges, according to reports of HCV genotyping (9, 10). It is noteworthy that all 4 patients had common variations of G to A at nt 145, C to T at nt 151, and G to A at nt 241. These variations were not seen in other sequences of various genotypes. Therefore, we synthesized the RNAs coding the HCV 5' noncoding region with

different nucleosides at nt 145 and nt 151 (standard, NCR145G151C; variants, NCR145A151T, NCR145G151T, and NCR145A151C). The nucleoside type at nt 241 was A in both RNAs, as the region around it commonly shows different sequences among HCV genotypes (9). The RNAs synthesized in various copy numbers/ml were quantified with CAP/CTM version 1. Synthesized RNAs of NCR145G151C and NCR145G151T at 10¹⁰ copies/ml were quantified as 3.9 log IU/ml and 3.7 log IU/ml, respectively, with CAP/CTM version 1, while the RNAs of NCR145A151T and NCR145A151C were undetectable. It was reported that the specific types of nucleosides at nt 107, nt 165, and nt 206 are associated with underestimation of HCV RNA levels, as for HCV genotype 2 (6). However, those features were not observed in the sequences of the present patients.

Reassessment of serum HCV RNA by CAP/CTM version 2. The levels of HCV RNA were also reassessed by CAP/CTM version 2 for the serum samples with discrepant quantification results. The samples resulted in equivalent levels of HCV RNA by both CAP/CTM version 2 and ART.

Analysis of nucleotide variation at nt 145 in the HCV 5' noncoding region retrieved from GenBank. A total of 1,090 HCV sequences were retrieved from GenBank, exclusive of repetitive sequences, including 472 of genotype 1a, 466 of genotype 1b, 73 of genotype 2, 34 of genotype 4, and 3 of genotype 5. The nucleotide type at nt 145 is G in most sequences and C in 17 sequences among HCV genotype 1a. Only one sequence among the HCV genotype 1b sequences had the specific nucleotide variation type of A at nt 145. All the sequences among HCV genotypes 2 to 5 in GenBank had G at nt 145.

DISCUSSION

The present study is the first report of patients with HCV genotype 2a, in whom CAP/CTM version 1 failed to detect HCV RNAs despite detection of a high viral load by ART. If such patients are erroneously considered to have spontaneous viral clearance or autoimmune hepatitis with positive anti-HCV antibody, the opportunity of their receiving critical antiviral therapy will be lost. Occult HCV infection, defined as detection of HCV RNA in liver tissue or peripheral blood mononuclear cells with constantly undetectable HCV RNA in serum (11), might involve such cases in which HCV RNA is undetectable with CAP/CTM version 1. Reassessment of HCV RNA with ART or quantification of HCV core antigen is desirable in cases in which HCV is not detected by CAP/

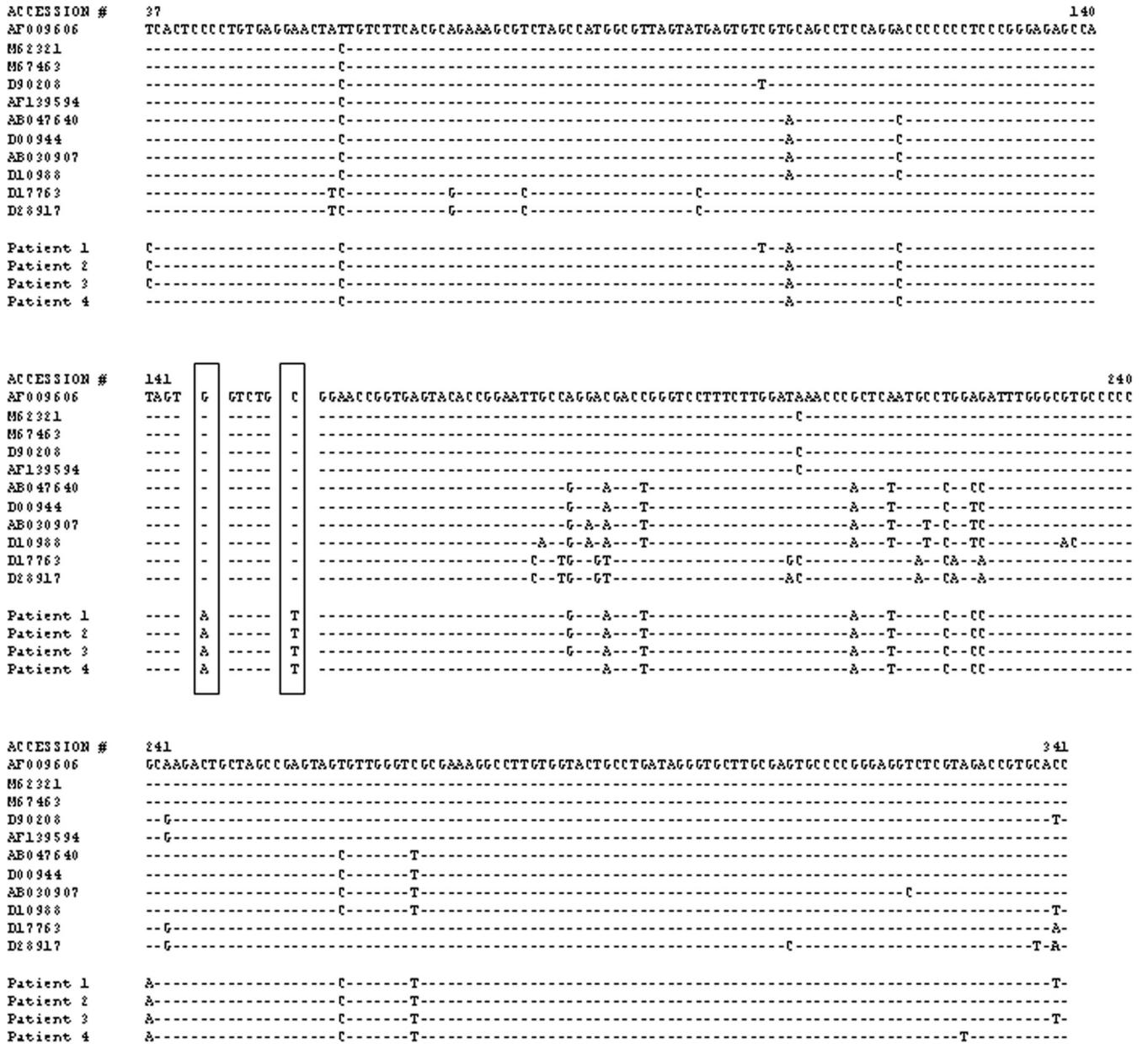


FIG 2 Sequence alignment of the HCV 5' noncoding regions of various HCV genotypes and the patients in this study. The sequence of HCV-H77 (GenBank accession number AF009606) was used as a reference; position numbers are given at the top. Dots indicate identical nucleosides. Sequence alignment of HCV 5' noncoding regions contains genotype 1a of HCV-1 (M62321) and HCV-H (M67463), genotype 1b of HCV-J (D90208) and HCV-N (AF139594), genotype 2a of JCH-1 (AB047640) and HC-J6 (D0944), genotype 2b of HCV-MA (AB030907) and HCV-J8 (D10988), and genotype 3a of HCV-NZL1 (D17763) and HCV-K3A (D28917). Sequences of patients 1 to 4 are aligned at the bottom.

CTM version 1. CAP/CTM version 2 is also helpful for the reassessment, if available.

In the present study, we investigated the characteristics of the patients whose serum HCV RNA was undetectable by CAP/CTM version 1. They were all male patients in their forties or fifties. We focused on the sequences of the HCV 5' noncoding region, targeted by CAP/CTM and ART, and compared their sequences with those of the representative strains of HCV genotypes 1a, 1b, 2a, 2b, and 3a. It is noteworthy that all of the patients had genotype 2a HCV and exhibited the same variations at nt 145 and nt 151. Quantification of synthesized RNA with these specific variations

at nt 145 and nt 151 confirmed that the variation at nt 145 caused the undetectability of HCV RNA by CAP/CTM version 1.

To find patients whose serum HCV RNA was undetectable by CAP/CTM version 1, the present study included consecutive patients who were positive for anti-HCV and admitted for further examination or therapy for liver-related disorders. We enrolled these patients because they were supposed to suffer from chronic liver diseases due to HCV, although their HCV RNA levels were not routinely assessed. According to the genotype distribution among Japanese patients with chronic hepatitis C, we assumed that two-thirds of these patients would be infected with genotype

1b of HCV, one-third with genotype 2, and a few with genotype 3. In the present study, we determined the HCV genotypes of all the enrolled patients, and the frequency of these specific variations was 0.45% among those patients. According to a search in GenBank, only one sequence of HCV genotype 1b had the specific nucleotide variation type of A at nt 145 among the sequences of HCV genotypes 1 to 5 (0.092%), suggesting a very low frequency of this specific variation.

In conclusion, in the present study we evaluated patients with HCV genotype 2a whose serum HCV RNA was undetectable with CAP/CTM version 1 despite detection of a high viral load by ART. Specific nucleotide variation at nt 145 was found to be responsible for the undetectability of HCV RNA by CAP/CTM version 1. Reassessment of HCV RNA with ART or quantification of HCV core antigen is desirable in cases in which HCV is not detected by CAP/CTM version 1. When it is available, CAP/CTM version 2 is also helpful for reassessment.

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We declare that we have no conflicts of interest.

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