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Stability of Hepatitis C Virus RNA in Blood Samples by TaqMan Real-Time PCR

Kenan Sener,[1]* Mehmet Yapar,[1] Orhan Bedir,[1] Cem Gül,[2] Ömer Coskun,[2] and Ayhan Kubar[1]

[1]Department of Medical Microbiology, Gulhane Military Medical Academy, Ankara, Turkey
[2]Department of Infectious Diseases, Gulhane Military Medical Academy, Ankara, Turkey

The storage conditions of blood samples for reliable results are very important in hepatitis C virus (HCV) RNA amplification tests used in routine HCV analyses. According to many studies, storage conditions could affect the RNA stability for HCV RNA detection. We have studied HCV RNA stability in blood samples stored at 4°C. Nineteen blood samples containing different HCV RNA levels were stored at 4°C and they were then analyzed by TaqMAN real-time PCR method. HCV RNA levels remained almost stable (100%) at least for five weeks at this storage condition. However, among them, the stability period was up to 11 weeks in two of the samples. As with these findings, there was a slightly significant correlation between the positivity time and the beginning HCV RNA levels ($r = 0.474$, $P = 0.040$). We conclude that, blood samples can be stored at 4°C for five weeks without any significant difference in detected HCV RNA level by using TaqMan real-time PCR. J. Clin. Lab. Anal. 24:134–138, 2010. © 2010 Wiley-Liss, Inc.

Key words: stability of HCV RNA; TaqMan; real-time PCR

INTRODUCTION

Hepatitis C virus (HCV) is an enveloped single-stranded RNA virus that is a member of *Flaviviridae* family (1). HCV infection is endemic worldwide with high prevalence rates, being the primary etiologic agent of transfusion-associated hepatitis (2). Furthermore, presence of HCV RNA has been shown to be correlated with the chronic hepatitis (3–5). Therefore, detection of HCV RNA is very important in blood as a screening and diagnostic test.

Quantification of HCV RNA in serum is used to predict treatment response and to monitor antiviral therapy. For this purpose, several assays have been described and some of these have been commercialized (6–8). These assays are based on different techniques. Especially, nucleic acid amplification techniques are being used in transfusional setting recently (9,10). The quality of these assays depends on the sensitivity and reproducibility of the amplification procedure, the choice of primers, the efficiency of the isolation of nucleic acids, and handling of blood samples before nucleic acid isolation. The effects of handling of blood samples to HCV viral load analyses have been studied for the COBAS Amplicor HCV-PCR, branched DNA signal amplification, and HCV Nucleic Acid Sequence Based Amplification-QT (NASBA-QT) assay but not for the real-time TaqMan PCR (11–15).

The stability of HCV RNA in samples from different origins (plasma, blood, and serum) in the presence of different preservatives (citrate, EDTA, etc.) at different storage temperatures (−70, −20, 4°C, room temperature, etc.) was reported in several studies (11–20). These studies were performed at different handling conditions such as transfusion settings, plasmapheresis, etc., and using different analytical methods (21). Results of these studies have been useful in developing some general guidelines for handling specimens for HCV RNA quantification. For example, it is generally agreed that HCV RNA loss can be minimized by separating serum samples within 2–4 hr of clot formation followed by short-term storage ($\leq$5 days) at 4°C or long-term storage

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*Correspondence to: Kenan Sener, Department of Virology, GATA, Turkey. E-mail: tabipks@yahoo.com, mehmet_yapar@hotmail.com

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(up to 1 year) at $-20^{\circ}C$ or lower (16,18,22–24). However, many of earlier studies were performed using semi-quantitative methods and/or tested limited numbers of specimens (≤ 12 samples) (16–24).

In this study, we evaluated the stability of HCV RNA in 19 blood specimens without any preservative and any separation process stored at $4^{\circ}C$ with real-time TaqMan PCR.

MATERIAL AND METHODS

Samples

Blood samples from 19 HCV-RNA positive patients were collected in standard vacutainer tubes without anticoagulant. The samples were not centrifuged and serum was not separated into a new tube. The blood samples were stored at refrigerator at $4^{\circ}C$ during the study. Repeated RNA isolations were performed once a week and the specimens were immediately put into the refrigerator back after each sampling for RNA isolation.

Nucleic Acid Isolation and Real Time TaqMan PCR

All the isolation procedure was performed in a class II cabinet. Viral RNA was extracted from a 100 μl portion of the sample using acid-phenol-chloramphenicol method and was dissolved in 100 μl deioinized RNAse/DNAse free water.

Oligonucleotide Design

The oligos, primers and probe, used in this study were designed with OligoYap version 4.0, a recently developed software program (25). The OligoYap 4.0 can accurately detect one of the highly conserved regions of any nucleic material and is able to select the oligos particularly based on its own gene database of the sequences generated with those available from the EMBL, GenBank, and DDBJ databases (26). We analyzed them whether they have optimum real-time PCR assay conditions with OligoYap 4.0. In addition, this software program gave us the opportunity to analyze if there were any degenerative bases on primers and probe.

TaqMan Based One-Step RT Real-Time PCR Assay

The assay was performed in a CHROMO4 Real-time PCR Detector (BioRad, Hercules, CA) by using EZ RT-PCR kit (Applied Biosystem, Foster City, CA) that utilizes rTth DNA polymerase. Amplification was carried out in 25 μl reaction mixture containing 5 μl of target virus RNA, 4 pmol of each primer, 3 pmol of TaqMan probe, 0.2 mM of each dNTP (containing doubled dUTP), and 6 mM $MgCl_2$. Cycling conditions were as follows: 2 min at $50^{\circ}C$, 30 min at $60^{\circ}C$, 5 min at $95^{\circ}C$, and then 40 cycles of 15 sec at $95^{\circ}C$ followed by 1 min at $60^{\circ}C$.

Quantification

HCV RNA was amplified with the used primers designed with OligoYap 4.0., HCV P1, and HCV P2, and then the amplicon was cloned with TOPO-TA Cloning kit (Invitrogen, USA). This plasmid, called HCQ, was used for HCV RNA quantification. The serial dilutions of HCQ plasmid DNA were also used for determining the dynamic range of quantification. Standard plasmid concentrations were analyzed with spectrophotometry and OligoYap 4.0.

Statistical Methods

Statistical analysis (Kaplan–Meier analysis for probability of specimen's failure) and data transformations (log transformation, standard deviation and standard error, comparison of means and medians, percentile distribution) were performed with the SPSS 10.0. A difference was considered significant if the *P*-value was lower than 0.05.

RESULTS

Nineteen HCV RNA positive serum samples were assessed. The quantity of HCV RNA in these serum samples ranged from 4.1×10^4 to 7.6×10^8 copy/ml, with a mean of 1.3×10^6 copy/ml (6.84 log; SD = 1.379) at the time point 0.

All the samples were found positive for HCV RNA at least for five weeks (Table 1). The mean viral load ($\pm$SD) was 6.84 ± 1.38 log at the time point zero and 6.04 ± 2.09 log at fifth week. The positivity period was 9 weeks long in five of the samples and 11 weeks in only two of the samples (Fig. 1).

It was realized that there was a slightly significant correlation between the positivity time and the beginning HCV RNA levels ($r = 0.474$, $P = 0.040$). Beginning HCV RNA levels of two samples that were positive for 11 weeks were 6.80 logs and 7.72 logs, whereas positivity period was 10 weeks in the sample with the highest level (8.88 logs). The mean ($\pm$SD) beginning HCV RNA levels were significantly lower for the samples that were only positive for 5 weeks (6.19 ± 1.03) compared to the those that were positive more than 10 weeks (8.05 ± 0.98) ($P = 0.01$).

Decrease in HCV RNA values of each sample in the last week before the HCV RNA became undetectable was more than 1 log in the samples except three of them (sample numbers 6, 11, and 14 in Table 1).

TABLE 1. HCV RNA Levels (log)

Sample no.	Week 0	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	Week 7	Week 8	Week 9	Week 10	Week 11
1	6.80	6.78	6.81	6.69	6.88	6.85	6.81	6.80	6.69	6.69	5.40	4.95
2	7.72	7.61	7.64	7.48	7.56	7.45	7.34	7.15	6.95	6.61	6.15	5.83
3	8.88	8.80	8.88	8.88	8.88	8.92	8.74	8.81	8.15	6.91	4.83	ND
4	8.78	8.83	8.73	8.78	8.88	8.91	8.48	8.43	8.40	7.40	5.94	ND
5	7.69	7.56	7.56	7.45	7.61	7.56	7.56	7.40	6.80	5.88	ND	ND
6	6.88	6.80	6.78	6.76	6.69	6.80	6.45	5.91	ND	ND	ND	ND
7	4.88	4.80	4.80	3.88	3.56	3.61	2.76	2.80	ND	ND	ND	ND
8	8.85	8.86	8.76	8.83	8.95	8.94	6.90	ND	ND	ND	ND	ND
9	8.23	8.40	7.92	7.95	7.92	8.34	6.94	ND	ND	ND	ND	ND
10	5.56	5.56	5.56	5.56	5.56	4.56	3.80	ND	ND	ND	ND	ND
11	7.95	7.94	7.92	8.15	8.04	7.48	ND	ND	ND	ND	ND	ND
12	6.85	6.76	6.04	5.95	5.80	5.64	ND	ND	ND	ND	ND	ND
13	5.85	5.76	5.76	5.78	4.95	3.15	ND	ND	ND	ND	ND	ND
14	6.56	6.52	6.40	6.28	6.28	6.15	ND	ND	ND	ND	ND	ND
15	6.66	6.52	6.23	6.56	5.90	5.28	ND	ND	ND	ND	ND	ND
16	6.94	6.83	6.91	6.72	5.88	5.15	ND	ND	ND	ND	ND	ND
17	5.56	5.34	5.23	4.80	3.92	3.56	ND	ND	ND	ND	ND	ND
18	4.61	4.56	4.28	3.88	3.81	3.61	ND	ND	ND	ND	ND	ND
19	4.74	4.64	4.56	2.95	2.80	2.80	ND	ND	ND	ND	ND	ND

ND, not detected.

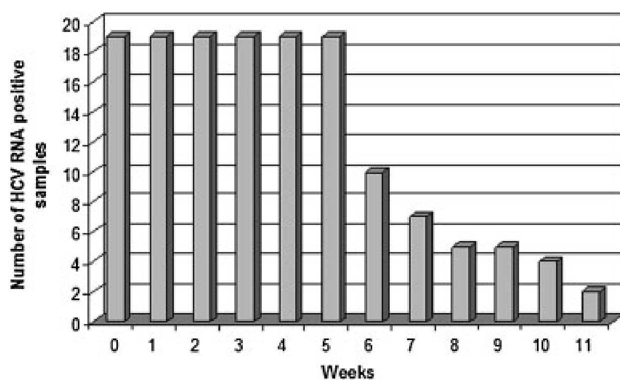

Fig. 1. Number of positive blood samples for HCV RNA in weeks.

TABLE 2. Estimated Probability of Specimen Failure by the Kaplan–Meier Analysis

Time (weeks)	Probability (%) of specimen failure
0–5	0
6	47.4
7	63.2
8–9	70.7
10	78.9
11	89.5

The probability of specimen failure over time at 4°C, as estimated by the Kaplan–Meier analysis, is shown in Table 2. During the five weeks' time when the HCV RNA level in all the samples could be determined, the probability of specimen failure estimated as zero. The percentage of failure varied from 47.4 to 89.5 between the weeks 6 and 11 (Table 2).

DISCUSSION

Detection of HCV RNA in blood is an important screening and diagnostic test. Furthermore, HCV RNA viral load has been described as important predictor of response to anti-HCV therapy. Unsuitable storage temperatures have been reported to affect HCV RNA stability and influence viral load measurement.

There are many studies on the stability of HCV RNA in plasma, serum, or blood samples. In these studies, the samples have been stored under different temperatures (4°C, room temperature, −20°C, −80°C, etc.) or aliquots of same sample have been prepared with different dilutions and under different temperatures (16–21). Different quantification methods have been used in the previous studies reported as well (11–15). To our knowledge, real-time PCR method has not been used for viral load quantification.

In a study, plasma samples taken from six HCV RNA positive patients have been stored at −80°C until the time of analysis. The samples were thawed at room temperature for 6 hr, and then aliquots were left at 4°C for 1, 2, 3, and 7 days before analysis. HCV RNA has been measured quantitatively through the COBAS Amplicor HCV Monitor test. Average HCV RNA levels have been reported as 5.49 logs at the time point 0, and 5.03 logs after 7 days (12). In our study, at the beginning

and at the fifth week, mean viral loads were 6.84 and 6.04, respectively.

In another study, HCV RNA levels were analyzed in 11 serum samples stored at 4°C for one month by using Quantiplex branched DNA method. The authors have reported that estimated probability of specimen failure according to Kaplan–Meier method was 18% (15). On the other hand, in the present study, the probability of specimen failure was 0% at the fifth week.

de Gerbehaye et al. have reported levels of HCV RNA in five HCV RNA positive patients' serum followed through the COBAS Amplicor HCV Monitor test for 96 hr. The average viral load at the starting point was 5.54 logs and its measurement on each sample remained at any time within 0.3 logs of the mean (14). In some of our samples, increase in HCV RNA values were noted, but these increases were within the assay variance of the used method. Interestingly, in most of the samples, HCV RNA levels decreased at least one log, one week before the HCV RNA became undetectable. The continuation of the stability until the last weeks of positivity may depend on particulate nature of the virus genome or the host's properties. Nevertheless, it requires further studies to speak clearly on this subject.

Beside to a commonly used COBAS Amplicor HCV Monitoring, another method used in the quantification of viral load is NASBA amplification. Damen et al. evaluated stability of HCV RNA level in blood samples stored at 4°C collected from four HCV RNA positive patients by NASBA amplification method. The authors showed that it was stable for 72 hr (13). In this study, we evaluated the stability of HCV RNA for a routinely used in-house real-time PCR test. The CV of this routinely used test in our laboratory is 14% for the viral loads in dynamic ranges (data not shown).

In another study, two dilutions (10^4 and 10^5 IU/ml) have been prepared from the HCV RNA positive plasma samples with citrate. Then, the dilutions were stored for 168 days at −70°C and followed through the Amplicor HCV Monitor test. It was determined that the half-life was similar in two dilutions (81 days for 10^4 IU/ml and 84 days for 10^5 IU/ml) (11). In our study, the stability of HCV RNA in blood samples stored at 4°C, which is practical for routine use, was inquired without transferring the serum of the samples to another tube. The test was repeated weekly until RNA level was not detectable in the samples. Additionally, our data suggests that level of viral load at the beginning may give an idea about the stability period in most of the samples. Low levels of HCV RNA in samples at the beginning caused continuing positivity lasting not more than 5 weeks, whereas samples with higher levels of HCV RNA in the samples at the beginning were stable more than 10 weeks.

The results of this study have showed that blood samples with HCV RNA levels up to 10^4 copy/ml can be stored at 4°C without any preservative for at least 5 weeks. HCV RNA quantification is a test often used in both monitoring treatment and screening blood donors. The obtained data are of the quality worth taking into consideration for especially the routine laboratories that have overload work and in the situations where repeated tests are required.

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