Interpretation of Positive Transcription-Mediated Amplification Test Results from Polymerase Chain Reaction–Negative Samples Obtained After Treatment of Chronic Hepatitis C

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The Siemens VERSANT® transcription-mediated amplification (TMA) assay is extremely sensitive for the detection of hepatitis C virus (HCV) RNA in serum. Eleven of 180 subjects in the Hepatitis C Antiviral Long-term Treatment against Cirrhosis (HALT-C) Trial who achieved polymerase chain reaction (PCR)-defined sustained virological response (SVR) at week 72 also had TMA-positive results from the same blood draw; six were positive on repeat testing. We report the follow-up on these 11 patients, and the reproducibility of TMA test results from PCR-negative samples in relationship to antiviral treatment outcome. Peginterferon and ribavirin treatment was initiated in 1145 prior interferon nonresponders with advanced hepatic fibrosis. Treatment was continued for 48 weeks if patients had undetectable HCV RNA by PCR at treatment week 20. Frozen serum samples from weeks 12, 20, 24, 48, and 72 were subsequently tested by TMA. Nine of the 11 patients returned for testing (median, 30 months after the week 72 visit), and all had undetectable HCV RNA by TMA and PCR. Among 759 PCR-negative samples obtained during treatment that were tested twice by TMA, 17% overall exhibited consistently positive results, and 21% exhibited inconsistently positive results. SVR was more likely if TMA was consistently negative than if consistently or inconsistently positive. With continued treatment, patients with inconsistently positive TMA results were more likely to become TMA-negative than TMA-positive ($P < 0.0001$). *Conclusion:* In PCR-negative samples, positive TMA results may indicate the presence of low levels of HCV RNA. However, because patients with positive TMA results may achieve SVR, management decisions during therapy should not be based on a single positive TMA test result. (HEPATOLOGY 2008;48:1412-1419.)

Abbreviations: BT/R, breakthrough/relapse; HALT-C, hepatitis antiviral long-term treatment against cirrhosis; HCV, hepatitis C virus; LLOD, lower limit of detection; PCR, polymerase chain reaction; SVR, sustained virological response; TMA, transcription-mediated amplification.

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The success of antiviral therapy for chronic hepatitis C is currently assessed by hepatitis C virus (HCV) RNA testing at least 24 weeks after completion of therapy.[1] Patients with undetectable virus at this juncture are considered to have achieved a sustained virological response (SVR), which has been associated with durable eradication of infection.[2-5]

In clinical trials, investigators have relied on qualitative HCV RNA assays [using polymerase chain reaction (PCR)] with lower limits of detection of approximately 50 to 100 IU/mL to establish undetectable HCV RNA levels during and after treatment.[1,6] Recently, the Siemens VERSANT HCV RNA Qualitative Assay, based on transcription-mediated amplification (TMA) technology, was approved by the US Food and Drug Administration for detection of HCV RNA in serum as evidence of active infection. The TMA assay has a lower limit of sensitivity of 5 to 10 IU/mL.[7-10] We recently described the performance of TMA for predicting SVR during the lead-in phase of the Hepatitis C Antiviral Long-Term Treatment against Cirrhosis (HALT-C) Trial, in which 1145 patients received combination pegylated interferon and ribavirin. Several studies, including our own, have demonstrated that positive TMA results at the end of treatment (week 48), despite negative results by PCR, identify subjects who subsequently relapse after cessation of treatment.[10-14]

Through extensive testing of our cohort, we have identified HALT-C Trial subjects with serum samples that were PCR-negative but TMA-positive during and after peginterferon and ribavirin treatment. The clinical implications of such results are uncertain, but potentially important, because clinicians are likely to base decisions regarding response to treatment on a single qualitative result, using the most sensitive assay available. In this analysis, we explore the clinical meaning of a positive TMA test in subjects with a negative PCR result for HCV RNA during or after peginterferon and ribavirin treatment.

Patients and Methods

Patients and Samples. The overall design of the HALT-C Trial has been described in detail.[15] Briefly, 1145 subjects with bridging fibrosis or cirrhosis who were nonresponders to prior treatment with interferon (with or without ribavirin) were retreated with peginterferon alfa-2a 180 μg/week and 1000 to 1200 mg/day of ribavirin during the lead-in phase of the trial. Subjects with undetectable HCV RNA using the Roche COBAS® Amplicor HCV Test, v. 2.0 assay (or PCR assay) at treatment week 20 received a total of 48 weeks of treatment with follow-up monitoring through week 72. SVR was defined as undetectable HCV RNA by PCR at week 72. Subjects with detectable HCV RNA at week 20 by PCR were deemed to be nonresponders to peginterferon/ribavirin, and treatment was discontinued at week 24. Clinical and other laboratory data were collected from all subjects according to standard procedures.[15] Serum samples obtained during the study were tested for HCV RNA at weeks 12, 20, 24, 48, 60, and 72. Frozen (−80°C) serum samples were subsequently tested by TMA as part of this study, including week 72 samples from all patients who had achieved SVR (defined by PCR). Patients who had achieved SVR but had positive week 72 samples by TMA were asked to return for repeat virological testing and clinical evaluation. The Institutional Review Boards of all participating institutions approved the study protocols, and written informed consent was obtained from all study subjects.

Virological Testing. Serum samples obtained from all subjects enrolled in the HALT-C Trial were frozen at each clinical site then shipped on dry ice and tested in real time at the University of Washington Virology Laboratory with both the quantitative Roche COBAS® Amplicor HCV Monitor Test, v. 2.0 assay [lower limit of detection (LLOD) 600 IU/mL] and, if negative, by the Roche COBAS® Amplicor HCV Test, v. 2.0 assay (or PCR assay, LLOD 100 IU/mL) as previously described.[16,17] HCV genotypes were determined with the INNO-LiPA HCV II kit (Siemens Medical Solutions Diagnostics, Tarrytown, NY).

The Siemens VERSANT® HCV RNA Qualitative Assay (or TMA assay) (Tarrytown, NY) was used to test thawed serum samples, according to the manufacturer's

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Table 1. Laboratory Data From Patients with PCR-Negative but TMA-Positive Results at Week 72

Subject	Baseline Ishak Score	GT	Baseline HCV RNA (x 10^6 IU/mL)	Baseline ALT	W72 ALT	W72 PCR	W72 TMA	W72 Aliquots TMA-pos	Months of F/U*	F/U PCR	F/U TMA	F/U Aliquots TMA-neg	F/U ALT
1	6	3a	1.34	64	33	–	+	1/3	23	–	–	2/2	34
2	3	1a	1.71	75	29	–	+	1/3	24	–	–	2/2	69
3	3	1a	0.48	96	52	–	+	1/4	46	–	–	2/2	38
4	6	1b	2.09	245	50	–	+	1/4	46	–	–	2/2	33
5	3	1b	0.79	98	50	–	+	1/4	47	–	–	2/2	31
6	3	1b	6.86	80	23	–	+	2/4	23	–	–	2/2	21
7	3	2	0.61	215	57	–	+	2/4	37	–	–	2/2	33
8	2	3a	1.69	39	35	–	+	2/4	LT F/U	LT F/U	LT F/U	LT F/U	LT F/U
9	2	1a/1b	11.4	50	32	+/–†	+	3/3	20	–	–	2/2	32
10	5	1a	1.06	36	25	–	+	2/2	30	–	–	2/2	23
11	2	1a	8.09	78	41	–	+	2/2	LT F/U	LT F/U	LT F/U	LT F/U	LT F/U

*Months of follow-up after week 72 (W72) visit.

†This subject had detectable serum HCV RNA by qualitative PCR (Amplicor) but not by quantitative PCR (Monitor) at his W72 visit. However, a repeat blood draw was negative by qualitative PCR shortly thereafter and he was considered an SVR. Samples from weeks 24, 36, 48, and 60 were also PCR-negative.

GT, HCV genotype; ALT, alanine aminotransferase (IU/mL); W72, week 72; PCR-, polymerase chain reaction-negative, defined as <100 IU/mL; TMA-, transcription-mediated amplification-negative, defined as <5 IU/mL; LT F/U, lost to follow-up.

instructions, except that five additional negative controls (HCV-seronegative serum samples) and an additional low-positive (sensitivity) control were used in each run of 88 patient samples. All stages of testing by this assay—sample preparation, target amplification, and amplicon detection—were performed within a single tube as described previously.[10] Briefly, the 5′ untranslated region of the HCV genome was amplified under isothermal conditions. Chemiluminescence of two differentially modified acridinium ester molecules attached to different probes allowed for the simultaneous detection of internal control and HCV RNA targets, as measured in relative light units. Each test result was considered valid if the internal control result was reactive for that sample. In our laboratory, HCV RNA is detected 100% of the time in serum by PCR at 100 IU/mL and TMA at 5 IU/mL.[10]

In this analysis, a large number of serum samples were tested twice by TMA in separate batches. The bulk of the TMA reproducibility data was obtained using PCR-negative serum samples because we had previously established that essentially all PCR-positive samples are also positive by TMA.[10] If PCR was negative and TMA positive, the results were considered "discrepant." If results of duplicate TMA tests from a single blood draw were the same (in other words, both results were negative or both results were positive), they were considered to be "concordant." If one TMA result was positive and the repeat TMA result was negative or vice versa, the results were considered to be "discordant."

Statistical Analyses. Chi-square tests were used to compare the percentage of positive or discordant tests among groups of patients or at different times. Analyses of results from multiple visits per patient were performed with the Cochran-Mantel-Haenstzel chi-square, stratified by visit number. Data were analyzed with SAS (Statistical Analysis Software, Cary, NC) version 9.1.

Results

Discrepant Results Obtained by PCR and TMA Testing After Completion of Antiviral Therapy. A total of 245 patients who had undetectable HCV RNA by PCR at the week 20 time point were also tested for HCV RNA at week 72 using the Roche COBAS® Amplicor test. One hundred eighty (70%) of these patients had undetectable HCV RNA using this assay and were deemed sustained virological responders. The TMA assay was positive on previously unthawed week 72 aliquots from 11 of these 180 patients (6.1%) (Table 1). This discrepancy could reflect the difference between the detection limits of the two assays (5 IU/mL for the TMA versus 100 IU/mL for the PCR test).[10] Alternatively, the discrepancy could have been caused by false-positive testing for HCV RNA by TMA or false-negative testing by PCR. The 11 TMA-positive results were derived from blood samples drawn and processed at six HALT-C clinical sites and were obtained from four separate runs of the TMA assay. Results from 9 of 11 samples produced robust signals (relative light units) relative to the positive threshold. All 11 patients had responded promptly to combination therapy with at least a 2 $\log_{10}$ decrease of HCV RNA levels by week 12 (early virological response), with 5 of these 11 also TMA-negative at week 12. All 11 patients also had remained TMA-negative and PCR-negative for HCV RNA from week 20 through week 48, with the exception of two subjects who had single TMA-positive results during therapy (one at week 20 and another at

Table 2. TMA Reproducibility Results Among PCR-Negative Samples

TMA Results	Week 12 N (%)	Week 20 N (%)	Week 24 N (%)	Week 48 N (%)	Total N (%)
Among SVR					
Negative/negative	110 (78.6%)	68 (84.0%)	62 (89.9%)	85 (95.5%)	325 (85.8%)
Discordant	9 (6.4%)	8 (9.9%)	5 (7.2%)	4 (4.5%)	26 (6.9%)
Positive/positive	21 (15.0%)	5 (6.2%)	2 (2.9%)	0 (0.0%)	28 (7.4%)
Total	140 (100%)	81 (100%)	69 (100%)	89 (100%)	379 (100%)
Among BT/R					
Negative/negative	15 (21.4%)	37 (28.0%)	36 (36.4%)	60 (76.0%)	148 (39.0%)
Discordant	21 (30.0%)	41 (31.1%)	25 (25.3%)	10 (12.7%)	97 (25.5%)
Positive/positive	34 (48.6%)	54 (40.9%)	38 (38.4%)	9 (11.3%)	135 (35.5%)
Total	70 (100%)	132 (100%)	99 (100%)	79 (100%)	380 (100%)

	% SVR According to TMA Reproducibility*				
TMA Results	Week 12 %	Week 20 %	Week 24 %	Week 48 %	Total
Negative/negative	88.0%	64.8%	63.3%	58.6%	68.7%
Discordant	30.0%	16.3%	16.7%	28.6%	21.1%
Positive/positive	38.2%	8.5%	5.0%	0.0%	17.2%

BT/R, breakthrough or relapse defined as reappearance of HCV RNA by PCR during or after completing peginterferon and ribavirin therapy.

*$P < 0.0001$ at all time points and overall for comparison of three groups and SVR, both as categories and as trend (Cochran-Mantel-Haenstzel chi-squared, stratified by time point).

week 48). Samples obtained from all 11 subjects at week 60 (off therapy) also were negative by TMA and PCR. Initial HCV RNA levels for the 11 patients ranged from 480,000 IU/mL to 11,400,000 IU/mL (median 1,690,000 IU/mL), and the patients were infected with various HCV genotypes, including 1a, 1b, 2, and 3a.

Additional aliquots of serum obtained at the week 72 time point from the 11 subjects were retested by the TMA assay within 3 months of the first TMA result (Table 1). All 11 subjects had sufficient serum for one repeat TMA test, whereas 9 of 11 individuals had sufficient serum available for three to four replicates to be performed. Six patients had a positive TMA result on repeat testing, and five had negative (discordant) results. The two samples with low relative light units initially were strongly positive on a second TMA test. Nine of these 11 patients had repeat clinical examination and HCV RNA testing by our laboratory at a median 30 months (range, 20-47 months) after the week 72 visit. On this return visit, all nine patients had HCV RNA undetectable by TMA testing of two separate aliquots of serum as well as by PCR (Table 1). One patient had an elevated alanine aminotransferase (patient 2, alanine aminotransferase = 69). Seven of these patients also had had negative repeat HCV RNA testing (usually by PCR) in local laboratories after week 72, and five of the seven had had negative tests on multiple occasions.

Reproducibility of TMA Results During Treatment. The finding that 11 patients with well-documented SVR at week 72 had serum that was positive by TMA, and that 6 of these 11 patients had positive results on repeat TMA testing led us to evaluate the reproducibility of the TMA assay. Potentially, the week 72 discrepant and discordant results could have been attributable to false-positive results in the initial TMA assay from laboratory or handling error. Alternatively, these samples might have contained extremely low levels of HCV RNA (<5 IU/mL), resulting in sampling variability with each test, as would be expected to occur near the limit of detection.

To evaluate the reproducibility of the TMA test, we first selected 30 serum samples randomly from patients with detectable HCV RNA by the Roche COBAS® Amplicor (PCR) test (LLOD, 100 IU/mL) but undetectable by the quantitative Monitor® test (LLOD, 600 IU/mL) for retesting with the TMA assay. All 30 samples had detectable HCV RNA on the first and repeat testing in separate runs by the TMA assay (data not shown). Thus, the reproducibility of the TMA test was 100% for samples containing HCV RNA levels that were greater than approximately 100 IU/mL.

As a next step, we performed replicate TMA testing on samples with HCV RNA-negative results by PCR (<100 IU/mL) (Table 2). A total of 815 randomly selected PCR-negative samples from various time points during therapy were therefore tested with the TMA assay on two separate aliquots on two separate occasions. Of these 815 samples, 274 had tested positive on initial TMA testing whereas 541 had tested negative.

To better understand the meaning of TMA test results among subjects with HCV RNA-negative results by PCR, we evaluated the virological outcome of these subjects according to the results of their duplicate TMA tests. In

all of these analyses, we reasoned that if TMA-discordant results were a consequence of laboratory or handling error, then they should not be associated with any particular virological pattern or outcome. Conversely, if discordant TMA results were attributable to low HCV RNA copy numbers close to the limit of detection, then discordant results might be expected to reflect a transition from HCV RNA consistently detectable to HCV RNA consistently undetectable by TMA as HCV infection cleared. Such a transitional state would also be predicted to become less common with longer duration of treatment and found less frequently in patients who achieved a clinical SVR.

We performed three analyses based on the results of this replicate TMA testing. For the first analysis, we evaluated the frequency of concordant or discordant TMA results among subjects who achieved an SVR and those who failed to achieve an SVR [in other words, breakthrough/relapse (BT/R), Table 2]. Only patients with known week 72 virological outcomes were included in the analysis. Among samples from patients with SVR, the overall rate of discordance (across treatment weeks 12, 20, 24, and 48) between the first and second TMA result was 6.9% (26/379) compared with 25.5% (97/380) among patients without SVR ($P < 0.0001$). For both groups, the rate of TMA-discordant and TMA-positive/positive results declined from week 12 to week 48. Finally, at each treatment week, TMA-discordant and TMA-positive/positive results were more common among patients with BT/R than among those with SVR ($P < 0.0001$).

We also examined the data set in a second way, by analyzing the ability of concordant and discordant TMA results to predict SVR (Table 2, bottom). TMA-negative samples that were negative on repeat testing were associated with a high rate of SVR (68.7% overall), with the highest SVR rate observed when the TMA results were negative early in treatment (for example, 88% at week 12). By contrast, samples that were repeatedly TMA-positive predicted a much lower rate of SVR (17.2% overall), where the likelihood of SVR was lower if samples were collected after a longer duration of therapy (range, 38.2% SVR at week 12 to 0% SVR at week 48). Finally, if the sample yielded discordant TMA results, the likelihood of achieving SVR was similar to that of samples with reproducibly TMA-positive results (21.1% overall).

For the third analysis, we tested the hypothesis that TMA-discordant virological results reflected a very low virus level or "phase" during therapy through which some patients passed en route to becoming HCV RNA-undetectable. To this end, we analyzed sequential virological results among patients who had a TMA-discordant result at week 12 with a subsequent week 20 result or had a TMA-discordant result at week 20 with a subsequent week 24 result (Fig. 1). For this analysis, a positive sample was defined as one that was PCR-positive or TMA-positive (singly or in duplicate) and a negative sample was defined as one that was TMA-negative (singly or in duplicate). We then determined whether a TMA-discordant result was more likely to be followed in time (during therapy) by a TMA-negative or positive result. The TMA-discordant result was more likely to be followed by a TMA-negative than positive result. Of 21 subjects with discordant results at week 12, 19 became HCV RNA-negative compared with two who became HCV RNA-positive at week 20. Likewise, of 32 subjects with discordant results at week 20, 24 became HCV RNA-negative compared with eight who became HCV RNA-positive at week 24 ($P < 0.0002$ and $P < 0.0047$, respectively, testing whether the proportion of TMA-negative and TMA-positive samples after TMA discordance differs from 50%). This pattern suggests that during peginterferon treatment and at concentrations of HCV RNA that are undetectable by PCR, the HCV RNA level declined from TMA-positive to TMA-discordant (very low levels) to TMA-undetectable.

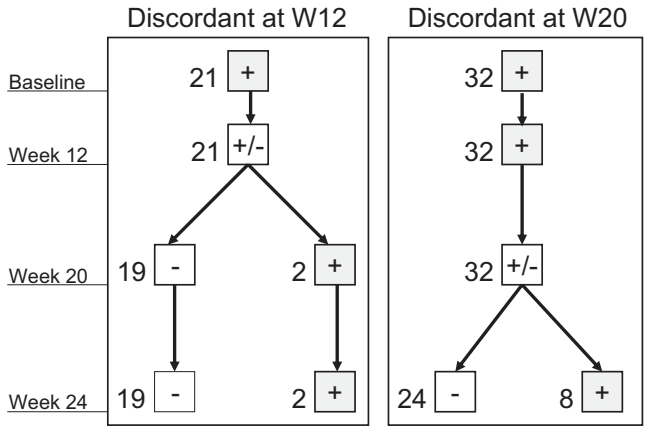

Fig. 1. Longitudinal virological results after discordant or inconsistent TMA results at week 12 or week 20 of treatment. Patients included in this analysis were those who: (left panel) had HCV RNA–positive results at baseline, had TMA-discordant (±) results at week 12, and subsequent virological results available at weeks 20 or (right panel) had HCV RNA-positive results at week 12, had TMA-discordant (±) results at week 20, and subsequent virological results available at week 24. Positive samples (+) were defined as those that were PCR-positive or TMA-positive (singly or in duplicate). Negative samples (−) were defined as those that were TMA-negative (singly or in duplicate). TMA-discordant or inconsistent results at weeks 12 or 20 were more likely to be followed by TMA-negative than TMA-positive results (19 negative versus 2 positive, $P < 0.0002$ and 24 negative versus 8 positive, respectively, $P < 0.0047$).

Discussion

The absence of detectable serum HCV RNA by PCR 6 months after completing therapy is the definition for

treatment success in hepatitis C patients receiving peginterferon and ribavirin therapy.[1] We and others have shown that the TMA assay is highly sensitive for HCV RNA detection[7-10] and that HCV RNA can be detected by TMA in samples that are HCV RNA-negative by standard PCR assays.[10-14,18] With increased TMA sensitivity, however, may come reduced specificity, and as TMA assays become increasingly available at clinical laboratories, the meaning of a positive TMA test during and after treatment should be understood before clinical decisions are based on such a result. In the current study, we examined the long-term virological outcome of subjects receiving 48 weeks of peginterferon and ribavirin with a positive TMA result at week 72. We further investigated the reproducibility of TMA results among serum samples that were negative by a qualitative PCR test (sensitivity, 100 IU/mL).

The most notable finding was that HCV RNA detectable by TMA but undetectable by PCR did not necessarily signify that a clinically meaningful serum level of virus was present. This conclusion was supported most strikingly by the observation of a true SVR in nine patients (as defined by long-term follow-up) in whom PCR was negative but TMA was positive 24 weeks after completing 48 weeks of peginterferon and ribavirin therapy. Consistent with their achieving an SVR, these patients had a prompt drop in HCV RNA on therapy, were repeatedly PCR-negative from week 20 through week 72, and had a significant decline in alanine aminotransferase activity from baseline to week 72. Most importantly, all nine remained HCV RNA-negative by both PCR and TMA after week 72. Although other investigators, using less sensitive methods for detecting HCV RNA, have described the presence of low-level HCV in serum and liver after successful peginterferon/ribavirin treatment,[2,5,19] in these nine patients, a positive TMA test did not signify a clinically meaningful recrudescence of HCV infection.

Because the TMA results at week 72 were shown to be consistently positive among several of the patients in the setting of apparent true SVRs, we systematically retested a large number of PCR-negative aliquots from patients who did or did not achieve SVR using the TMA assay (Table 2). At all time points tested, PCR-negative samples with consistently positive as well as inconsistently positive results by TMA were identified. It was notable that patients with BT/R had much higher rates of TMA-positive/positive or TMA-discordant results than patients with SVR, suggesting that a positive TMA result, even if discordant, accurately detects HCV RNA and predicts a lower likelihood of successful clearance with peginterferon and ribavirin treatment. Finally, at week 12 of therapy, patients with SVR had as high a proportion of consistently negative TMA results (78.6%) as did patients with BT/R at the end of therapy (76.0%). These results are consistent with the interpretation that the antiviral pressure of peginterferon/ribavirin treatment is more successful at eliminating circulating HCV RNA during therapy in those who will eventually achieve SVR compared with those who do not, and that such control may occur later and is ultimately inadequate in those who do not achieve SVR.

We have previously shown that, among patients with negative PCR results while on antiviral therapy, virological breakthrough or relapse was preceded by a positive TMA result in most but not all subjects.[10] Thus, for clinical guidance, knowing the reproducibility of TMA and the likelihood of SVR based on the TMA result is important for patient management. Among PCR-negative samples in this study, 16% of all samples tested twice by TMA exhibited inconsistent or discordant results. Over all time points, consistently negative TMA results led to a 69% likelihood of SVR, whereas consistently positive results led to a 17% likelihood of SVR. When TMA results were inconsistent on repeat testing, the overall likelihood of SVR was 21%, only slightly greater than in patients with consistent TMA-positive results. This observation supports the hypothesis that HCV RNA was present in the samples that exhibited inconsistently TMA-positive results.

Successful antiviral treatment usually results in progressively lower levels of HCV RNA with longer duration on therapy until the virus is no longer detectable. With a highly sensitive qualitative test such as TMA, we hypothesized that discordant or inconsistent results might follow reproducibly positive results and precede reproducibly negative results as viral levels declined. The patterns shown in Fig. 1 are consistent with this hypothesis. Patients were much more likely to have the sequence of TMA-positive followed by discordant followed by negative than they were to have the sequence of TMA-positive followed by discordant followed by positive.

Although we cannot exclude the possibility that laboratory or handling error could have contributed to some of the discordant results reported here, the patterns of TMA reproducibility observed in this study suggest that the bulk of the discordant results reflect low levels of HCV RNA in PCR-negative/TMA-discordant samples. Such a conclusion is consistent with our previous description of samples containing less than 5 IU/mL (from a defined HCV RNA standard) in which HCV RNA was detected inconsistently by TMA.[10] Although laboratory errors also could have contributed to the week 72 TMA-positive results in the 11 patients with PCR-defined SVR, all negative controls in those runs were negative, and no

other aberrant results were noted. The HALT-C virology laboratory has stringent procedures in place to minimize the possibility of contamination including the use of separate "clean" and "product" areas combined with a designated work flow plan that prohibits moving from the "product" to the "clean" area in a single day. Furthermore, these positive results were obtained on four separate runs, and contamination from neighboring wells could not have accounted for all of the results reported.

In clinical practice, serum samples are unlikely to be tested repeatedly by a qualitative test for HCV RNA, much less tested repeatedly by two qualitative tests as was done in this study. The HALT-C Trial, however, provided a unique opportunity to demonstrate the reproducibility of the TMA qualitative test in a large number of patients treated under a standardized protocol and whose serum samples were tested at a dedicated, single-site, virology laboratory. In the absence of another HCV RNA assay with 100% detection at 5 IU/mL, we cannot conclude definitively that unexpectedly and inconsistently positive samples by TMA resulted from very low levels of virus in the PCR-negative samples. However, we have presented evidence that inconsistent detection of HCV RNA by TMA was not random, as would have been expected with laboratory error. Rather, our data were most consistent with the hypothesis that inconsistent detection of HCV RNA in the TMA assay reflects the sampling variability that occurs in the presence of very low levels of the target molecule. Moreover, it suggests that patients successfully treated for HCV infection could potentially harbor extremely low levels of HCV RNA after treatment.

Highly sensitive assays such as TMA are being increasingly used to monitor response to therapy. Thus, detailed information on the limitations of these sensitive assays is important for therapeutic management. Some suggestions regarding the clinical use of TMA were discussed in a prior manuscript.[10] The TMA assay can detect the presence of HCV RNA among patients in whom a PCR assay is negative. However, a single positive TMA test result should be interpreted with caution, because, in our cohort of patients, a single positive TMA test did not necessarily signify nonresponse to treatment or failure to achieve SVR. Our previous finding that 2 of 19 patients (11%) with PCR-negative but TMA-positive results at the end of therapy (week 48) still achieved SVR[10] lends additional weight to this warning. Whether positive TMA results could potentially represent the detection of replication-incompetent HCV RNA in these patients is unknown. It is important to note that the added sensitivity of the TMA assay has been shown to provide useful information in other settings[10-14]; thus, we encourage additional studies to be performed in other cohorts. Nevertheless, based on our findings, we suggest that decisions regarding the clinical management of patients during hepatitis C antiviral therapy should be made differently if highly sensitive qualitative tests are used (LLOD 5-10 IU/mL), rather than less sensitive HCV RNA tests (LLOD 50-100 IU/mL). Here, we have examined the reproducibility of TMA among PCR-negative samples during and after therapy. Based on these results, we would specifically discourage the use of single TMA test results for treatment discontinuation decisions, as is currently recommended for PCR-based assays. Moreover, unexpected TMA-positive results may best be handled by careful clinical follow-up.

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