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Hepatitis C Virus (HCV) Core Antigen Assay To Detect Ongoing HCV Infection in Thai Injection Drug Users

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We evaluated a quantitative enzyme immunoassay (trak-C) for hepatitis C virus core antigen (HCV core Ag) by testing serum specimens from 820 injection drug users in Thailand with anti-HCV antibodies. The HCV genotypes in this population include genotypes 3 and 6, which have not been extensively tested with this assay. Among these specimens, 629 (76.7%) yielded positive results, with HCV core Ag concentrations predominantly spanning (35.7%) or above (58.2%) the measurable range of 1.5 to 100 pg/ml. To assess reproducibility, we retested 30 specimens representing six core Ag ranges; the mean coefficient of variation for each range was ≤9.7% (highest for 1.5 to 25 pg/ml). We also tested 204 specimens of the 820-specimen set for HCV RNA: while 146 (71.6%) were core Ag positive, 168 (82.4%) had detectable HCV RNA, of which 96% were typeable as genotype 3 (39%), 1 (31%), or 6 (26%) by nested reverse transcription-PCR. Among RNA-positive specimens, 86.9% had core Ag; 94% of the RNA negatives were core Ag negative. While there was no apparent bias for detecting core Ag representing the tested genotypes, median quantified results were higher for types 1a and 6 than for genotype 3 ($P = 0.01$); similarly, the median core Ag concentration was higher in HCV-human immunodeficiency virus-coinfected subjects than in HCV-monoinfected subjects. Our results demonstrated a good correlation between core Ag and HCV RNA in this population with high frequencies of genotypes 3 and 6. Because most core Ag concentrations were greater than those in the measurable range, we recommend a 10-fold dilution of the specimen before quantification. Reproducibility, low technical requirements, and high throughput should make this assay useful for clinical or research monitoring of HCV levels during active infection.

Hepatitis C virus (HCV) is an important human pathogen with global health effects. HCV infects up to 3% of the general population, with an estimated 3.9 million people infected in the United States and 170 million people infected worldwide (1, 30). HCV infections frequently persist and can lead to cirrhosis or hepatocellular carcinoma (27, 29).

The principal means of diagnosing HCV infection is by use of an enzyme-linked immunosorbent assay (ELISA) to detect anti-HCV antibodies in blood (2, 15). Supplemental testing is required to prove that the ELISA is not falsely reactive and to determine if the infection is ongoing or resolved. Whereas supplemental antibody tests (i.e., strip immunoassays) can be used to identify false-positive ELISA results, only the direct detection of viral RNA or antigen differentiates ongoing from resolved infections. Among direct detection tests, those that measure the quantity of RNA (or possibly antigen) can also be used to evaluate the likelihood of response to treatment and to monitor the treatment response (6, 13, 17). However, HCV RNA tests are technologically difficult and thus not practical for common use worldwide.

Recently, a test (trak-C; Ortho Clinical Diagnostics, Raritan, N.J.) was commercially developed to detect HCV core antigen (core Ag). Because this assay uses a standard ELISA format and is easy to perform, it might serve a worldwide role in HCV diagnostics. We evaluated its performance by studying specimens from a large population of injection drug users in northern Thailand, a setting with multiple HCV genotypes. We assessed the specificity and sensitivity of the core Ag assay by testing a subset of these samples with assays for HCV RNA and genotype.

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MATERIALS AND METHODS

Specimens. Serum specimens were collected from an intravenous-drug-use cohort in Chiang Mai, Thailand (19). The Opiate User Research project was initiated to determine sociodemographic, sexual, and drug-use risk factors for human immunodeficiency virus (HIV) infection among drug users in an area of northern Thailand adjacent to the Golden Triangle region. Enrolled subjects were selected from inpatients at a drug detoxification treatment center, i.e., 1,865 patients who had been treated for opiate and methamphetamine dependence between 1 February 1999 and 31 January 2000. Serum was collected from each subject upon enrollment and then tested for antibodies to HIV and HCV. For this study, we selected enrollment serum specimens that were repeatedly reactive for anti-HCV: 820 were selected for core Ag testing, and 204 of the 820-specimen set (selected from subjects with the most follow-up visits) were selected for qualitative HCV RNA detection.

Core Ag assay. HCV core Ag was detected or quantified by use of the trak-C assay performed according to the manufacturer's instructions.

HCV RNA assays. (i) Qualitative. To detect HCV RNA, we used the COBAS AMPLICOR HCV test version 2.0 (Roche Molecular Systems, Branchburg,

N.J.) according to the manufacturer's instructions. This assay has a limit of detection of 2.0 $\log_{10}$ IU of HCV genotype 1 RNA per ml of serum.

(ii) Quantitative. To determine the concentration of HCV RNA in serum, we used a quantitative reverse transcription (RT)-PCR assay (COBAS AMPLICOR HCV MONITOR version 2.0; Roche Molecular Systems) according to the manufacturer's protocols. This assay has a lower limit of quantitation of 2.78 $\log_{10}$ IU/ml.

Genotyping. Samples were genotyped by direct sequencing of RT-PCR products representing core- and E1-encoding regions (C/E1) of the HCV genome. For most RT-PCRs, we used RNA extracts prepared for the Roche qualitative assay. Otherwise, RNA was extracted from serum (100 μl) with the QIAamp viral RNA mini kit (QIAGEN, Valencia, Calif.) as per the manufacturer's recommendation, except that the serum volume was adjusted with phosphate-buffered saline (pH 7.4) to 140 μl before extraction. RT and first-round PCR were performed together with a 50-μl reaction volume containing 10 μl RNA, 1× PCR buffer, 0.4 μM forward (493S_H77, 5′-GCAACAGGGAACCTTCCT GGTTGCTC-3′) and 0.4 μM reverse (987R_H77, 5′-CGTAGGGGACCAGTT CATCATCAT-3′) primers (18), 0.2 mM deoxynucleoside triphosphates, 1.5 mM $MgCl_2$, 10 U of RNase inhibitor (RNaseOUT), 80 U of Moloney murine leukemia virus reverse transcriptase, and 1.5 U of Platinum Taq polymerase. All reagents and enzymes were purchased from Invitrogen (Carlsbad, Calif.). This reaction was incubated at 42°C for 40 min; 94°C for 2 min; 30 cycles of 94°C for 15 s, 60°C for 15 s, and 72°C for 30 s; and 72°C for 5 min. The second round of PCR was performed with 2 μl of first-round product in a 50-μl reaction volume containing the same PCR components as those contained in the first round except for the Moloney murine leukemia virus reverse transcriptase and primers. Primers for second-round amplification were forward (502S_H77, 5′-AACCTT CCTGGTTGCTCTTTCTCTAT-3′) and reverse (975R_H77, 5′-GTTCATCAT CATATCCCATGCCAT-3′). Second-round incubation conditions were the same as those in the first round, excluding the RT (42°C) step. Products were analyzed by electrophoresis in a 1.5% agarose gel stained with ethidium bromide. PCR products were purified with QIAquick PCR purification kits (QIAGEN) prior to sequencing.

Sequencing and phylogenetic analysis. Nucleotide sequences of purified PCR amplicons were determined by use of a PRISM version 3100 automated sequencer (ABI, Foster City, Calif.). Primer sequences were removed prior to analysis. Sequences were assembled and analyzed by using BioEdit version 5.0.9 (Tom Hall; available from the author at http://www.mbio.ncsu.edu/BioEdit/bioedit.html) and aligned by using ClustalX version 1.83 (9). Phylogenetic trees were inferred using algorithms from the DNADIST, NEIGHBOR, SEQBOOT, and CONSENSE programs in the PHYLIP suite version 3.5p (5) and implemented in Nimble Tree version 2.5 (S. Ray; available from the author at http://sray.med.som.jhmi.edu/RaySoft). After distance matrices under the F84 model were calculated, the neighbor-joining algorithm (20) was used to infer trees, which were rendered with TreeView (14, 23).

Statistical analysis. The nonparametric Kruskal-Wallis test was utilized to test for differences in core Ag concentrations among different genotypes. For comparison of core Ag concentrations in HCV-monoinfected subjects and HCV-HIV-coinfected subjects, the nonparametric Mann-Whitney test was utilized. Coefficient of variation (CV) was used as a measure of core Ag assay precision.

RESULTS

Characteristics of subjects. The study represented 820 subjects, 768 males (93.4%) and 52 (6.3%) females. The mean ages were 31 years for males and 35 years for females (standard deviations, 9 and 14 years, respectively); 306 (37.2%) subjects were HIV infected, while 514 (62.5%) were anti-HIV negative.

Core Ag in anti-HCV-positive specimens. We tested 820 specimens with the trak-C assay, 629 (76.7%) of which yielded positive results (Table 1). Among the core Ag-positive samples, 223 (35.7%) had results within the stated measurable range of 1.5 to 100 pg/ml. Most (64.4%) core Ag concentrations were outside the measurable range: 39 (6.2%) were below the lower limit of quantitation, and 367 (58.2%) were above the upper limit of quantitation. Table 2 includes proportions of the 630 positive results that were within the HCV core Ag concentration ranges of 1.5 to 25, 26 to 50, 51 to 75, and 76 to 100 pg/ml and those represented by two ranges above the measurable range (101 to 125 and 126 to 150 pg/ml).

TABLE 1. Direct detection of HCV in serum specimens from Thai subjects with antibodies to HCV

Specimen characteristic	No. of specimens tested	Assay	No. (%) of specimens positive
All	820	HCV core Ag, quantitative	629 (76.7)
Selected for HCV RNA testing	204	HCV core Ag, quantitative	146 (71.6)
		HCV RNA, qualitative	168 (82.4)

Interassay precision of core Ag assay. Thirty specimens were randomly selected to represent six ranges of core Ag concentration (Table 2). A second aliquot of each sample was then retested by the same operator on a subsequent day. Core Ag data were highly reproducible across all six ranges, with the highest mean CV (9.7%) among results for the lower end of the measurable range. The mean CV for the 51- to 75-pg/ml range was very low, at 1.67%. While the mean CV was <9% for the two highest ranges, it is not known if the values for the 101- to 150-pg/ml ranges were accurate because such results are greater than the upper limit of quantitation.

To characterize the interassay precision of external controls and core Ag calibrators, we calculated the CVs for these reagents over 11 runs conducted by one operator over 11 days (Tables 3 and 4). Over these 11 runs, the plate validity criteria were met, and the mean linear squared regression (r^2) value was 0.99. The CVs for the low- and high-positive controls, respectively, were 14.34 and 6.51% (Table 3). Analysis of interassay CV of core Ag calibrators ranged from 0.71 to 42.6%. The highest CV was 42.6% for the 1.5-pg/ml calibrator, and it should be noted that the calculated mean core Ag concentration of this calibrator was >50% off from the projected value (Table 4).

Dilution analysis: specimens with high concentrations of core Ag. As noted above, the majority of core Ag-positive samples yielded values greater than the upper limit of quantitation. To determine the core Ag concentration in such samples more accurately, we diluted and then retested five samples that had yielded values between 216 and 242 pg/ml (Table 5). As recommended by the manufacturer, these samples were tested after a 10-fold dilution in core Ag-negative human serum (provided in trak-C kits); calculated core Ag concentrations ranged from 568 to 1,110 pg/ml.

HCV core Ag versus HCV RNA. To assess the sensitivity and specificity of the core Ag assay, we further analyzed 204 samples from the 820-specimen set by testing for HCV RNA with a qualitative assay (Table 6). Among HCV RNA-positive specimens, 87% yielded positive results with the core Ag assay. The core Ag assay yielded negative results with 94% of the HCV RNA-negative specimens.

To determine if the 22 core Ag-negative and HCV RNA-positive results represented a sensitivity differential, we tested 21 samples (that had sufficient volume) with a quantitative RNA assay (Table 7). HCV RNA was quantified in 14 samples

TABLE 2. Run-to-run precision: stratified by ranges of HCV core antigen assay concentration[a]

Range of HCV core Ag concn (pg/ml)	% of specimens in range[a]	Specimen	HCV core Ag (pg/ml)				CV (%)	Mean CV (%)
			1st run	2nd run	Mean	SD		
1.5–25	18	2536	2.1	1.7	1.9	0.28	15	9.7
		2530	8.3	7	7.7	0.92	12	
		2515	9.1	7.9	8.5	0.85	10	
		2387	19.4	18.7	19.1	0.49	3	
		2438	22.1	19.4	20.8	1.91	9	
26–50	8.3	2456	35.8	30.6	33.2	3.68	11	6.8
		2543	38.7	43	40.9	3.04	7	
		2481	41.6	45.4	43.5	2.69	6	
		2318	42	37.5	39.8	3.18	8	
		2555	46	46.9	46.5	0.64	1	
51–75	4.3	1166	52.8	53.2	53	0.28	1	1.67
		0248	56.1	55.6	55.9	0.35	1	
		2521	61.3	59	60.2	1.63	3	
		2557	74.1	70.4	72.3	2.62	4	
		2414	74.5	75.4	75	0.64	1	
76–100	5.1	2392	76.4	81.6	79	3.68	5	6.88
		2258	80.1	78	79.1	1.48	2	
		2042	84.3	92.5	88.4	5.8	7	
		2274	86.8	78.9	82.9	5.59	7	
		2336	96.3	78.3	87.3	12.7	15	
101–125	4.5	2436	102.2	92.9	97.6	6.58	7	7.77
		2498	102.4	91.7	97.1	7.57	8	
		2422	107.6	99.4	103.5	5.8	6	
		2243	114	97.2	105.6	11.9	11	
		2235	117	105.3	111.2	8.3	7	
126–150	4.0	2256	127.7	103.3	115.5	17.3	15	8.57
		1787	129.7	129.1	129.4	0.42	0	
		829	136.3	158.3	147.3	15.6	11	
		1037	138.5	157.4	148	13.4	9	
		2499	146.9	131.2	139.1	11.1	8	

[a] Among 629 HCV core Ag-positive specimens in Table 1, 44.0% are represented in the ranges in this table (i.e., 1.5 to 150 pg/ml).

(range, 3.0 to 4.74 $\log_{10}$ IU/ml), was detected but not quantifiable in 2 samples, and was not detected in 5 samples.

Two samples were core Ag positive and HCV RNA negative. Insufficient volume precluded additional testing to determine if these results could be reproduced or interpreted by analysis of results from other testing.

Correlation of HCV genotype with core Ag data. We correlated genotyping data with core Ag results (Table 8). Among the 168 HCV RNA-positive samples, 161 were typeable. Genotype 3 was the most prevalent (39% of samples), followed by genotypes 1 (31%) and 6 (26%). The order of subtype prevalence was 3a (25%), 1a (22%), 3b (14.3%), and 1b (8.9%). It should be noted, however, that we included unclassified subtypes (such as 7c, 9b, and 9c) with genotype 6 (14, 22). This type distribution is consistent with previously reported data for this population and region of Thailand (4, 8, 10, 26). In addition, higher concentrations of core Ag were observed for persons who had type 1a or 6 than those with other genotypes ($P = 0.01$) (Table 8).

The genotype could not be determined for seven specimens, even though C/E1 RT-PCR is generally more sensitive (reproducibly detects less than 1.4 $\log_{10}$ IU/ml; unpublished data) than the Roche qualitative assay. C/E1 RT-PCR was attempted four times for each specimen: twice with RNA ex-

TABLE 3. Characterization of core Ag external controls over 11 runs[a]

Control	Acceptable core Ag concn (pg/ml)	Core Ag concn (pg/ml)		CV (%)
		Mean	SD	
Negative	≤1.1	0	0	0
Low positive	3.0–12.7	4.7	0.67	14.3
High positive	20.5–44.5	26.7	1.74	6.5

[a] All three controls were valid per quality control criteria for 11 runs.

TABLE 4. Characterization of core Ag calibrators over 11 runs

Calibrator concn (pg/ml)	Calculated concn (pg/ml)		CV (%)
	Mean	SD	
0	0	0	0
1.5	0.96	0.41	43
5	5	0.63	13
15	15.73	0.65	4.11
50	52.10	0.83	1.6
100	98.91	0.70	0.71

TABLE 5. Dilution analysis of five samples with HCV core Ag concentrations greater than upper limit of quantitation

Specimen	HCV core Ag concn (pg/ml)	
	Undiluted specimen	10-fold-diluted specimen[a]
1	242	671
2	237	1,110
3	228	600
4	216	722
5	216	568

[a] Calculated value.

tracted for the Roche qualitative assay and twice with RNA extracted from a new aliquot of serum. While three specimens yielded amplicon from the Roche assay extract (but not from newly extracted RNA), nucleotide sequence data were insufficient for deducing genotype; two of these specimens (Table 7, specimens 12 and 13) had quantifiable HCV RNA, and the third specimen (specimen 15) had HCV RNA at less than the quantifiable concentration. Three other specimens did not yield an RT-PCR product from either type of RNA extract; their Roche quantitative results were detected but not quantifiable (Table 7, specimen 16) or not detected (specimens 20 and 21). Core Ag was detectable in the seventh nontypeable specimen at less than the lower limit of quantitation, but insufficient volume precluded HCV RNA quantitation.

Core Ag and HCV-HIV coinfection. It has been reported that HCV RNA levels are higher in HCV-HIV-coinfected individuals than in HCV-monoinfected individuals (24, 25). Therefore, we analyzed median core Ag concentrations among mono- and coinfected subjects to determine if core Ag levels differed. Of 820 HCV antibody-positive subjects, 306 (37.2%) were HIV positive, 514 (62.5%) were HIV negative, and 2 (0.24%) had unknown HIV status. Median core Ag concentrations in HCV-HIV-coinfected subjects were significantly higher ($P < 0.01$) than those in HCV-monoinfected subjects (Table 9).

DISCUSSION

Molecular methods based on detection of HCV RNA are the "gold standard" for determining viral load and genotype. Accurate determinations of HCV RNA concentrations and HCV genotype in blood have proven clinical utility (7, 16).

TABLE 6. Detection of HCV core Ag and HCV RNA in 204 specimens selected for HCV RNA testing

Result of HCV core Ag assay[a]	No. of specimens with HCV RNA result[b]	
	Positive	Negative
Detected	146	2
Not detected	22	34

[a] Quantitative assay. As an estimate of sensitivity, HCV core Ag was detected in 146 (87%) of 168 HCV RNA-positive specimens (95% confidence interval, 81.8 to 92.0%). As an estimate of specificity, HCV core Ag was not detected in 34 (94%) of 36 HCV RNA-negative specimens (95% confidence interval, 81 to 99%).

[b] Qualitative assay.

TABLE 7. Analysis of specimens negative for HCV Core Ag and positive for HCV RNA: HCV RNA concentrations and genotypes

Detection level of HCV RNA and specimen	HCV RNA ($\log_{10}$ IU/ml)	Genotype[a]
Quantifiable		
1	4.74	3a
2	4.39	3b
3	4.30	3b
4	4.17	3a
5	3.87	1a
6	3.77	1a
7	3.52	3a
8	3.52	3a
9	3.33	3a
10	3.25	1b
11	3.21	6
12	3.18	NT
13	3.06	NT
14	3.00	1b
Below lower limit of quantitation		
15	<2.77	NT
16	<2.77	NT
ND[b]		
17	ND	1a
18	ND	6
19	ND	6
20	ND	NT
21	ND	NT

[a] NT, nontypeable: see text for explanation.

[b] ND, not detected. HCV RNA was not present or the concentration was below the limit of detection (not known).

Determination of HCV RNA levels permits assessment of HCV dynamics during antiviral therapy and helps predict the potential for response. The trak-C assay was developed as an alternative method to monitor HCV levels during treatment and found to correlate well with RNA assays (3, 11, 28). The assay is also easier to use than HCV RNA assays and thus

TABLE 8. Ranges of HCV core Ag concentrations among 168 samples containing HCV RNA, stratified by genotype and subtype

Genotype and subtype	No. (%) of specimens	Core Ag concn (pg/ml)	
		Median	IQR[a]
1			
Total	52 (31)	156.7	15.8–242.7
1a	37 (22)	206[b]	47.8–245.1
1b	15 (8.9)	36	1.1–184.3
3			
Total	66 (39)	58.9	8.7–220.8
3a	42 (25)	55.2	8.3–207.8
3b	24 (14)	72.7	8.1–222
6 (total)	43 (25)	220.1[b]	65.5–252.1
NT[c]	7 (4)	ND[d]	ND

[a] Interquartile range.

[b] $P = 0.01$ overall; $P < 0.10$ for results for genotype 6 versus those for subtypes 1b, 3a, and 3b; $P > 0.10$ for genotype 6 versus 1a (comparisons made using Kruskal-Wallis test with Bonferroni correction for multiple comparisons). Results were not altered after log transformation of core Ag values.

[c] NT, nontypeable.

[d] ND, not determined.

TABLE 9. Comparison of HCV core Ag concentrations among 820 specimens, stratified by HIV infection status[a]

HIV status	No. (%) of specimens	Core Ag concn (pg/ml)	
		Median	IQR[b]
Infected	306 (37.2)	100.6[c]	5.1–231.6
Not infected	514 (62.5)	40.5	0–219.6

[a] Results of testing for antibodies to HIV type 1 by ELISA and gel particle agglutination test or Western blot analysis.
[b] Interquartile range.
[c] $P < 0.01$ by Mann-Whitney test for results for HIV-infected specimens versus HIV-noninfected specimens.

could have an important role in less technologically developed regions of the world. Until now, however, analysis of this assay has not been performed with a population with a high prevalence of genotypes 3 and 6, which are endemic in Asia.

In this study, we evaluated the trak-C assay, which has low technical requirements (standard ELISA format), allows high throughput (86 specimens/run), and is relatively inexpensive (compared to RNA assays). This assay reliably detected HCV core Ag in serum. In addition, several other aspects of this study are noteworthy. Correlation was good (88.2%) between qualitative detection of core Ag and HCV RNA. Core Ag- and RNA-negative results likely represented subjects with resolved HCV infection. Discrepancies between the assays were due primarily to sensitivity differences. While the Roche qualitative HCV RNA assay's limit of detection is 2.0 $\log_{10}$ IU/ml, that of the core Ag assay is 1.5 pg/ml; the latter concentration corresponds to 4.1 $\log_{10}$ IU/ml (3) or 5.1 $\log_{10}$ IU/ml, if one assumes the presence of 1.8 virions per IU (21) and 180 core molecules per virion. The assay also was reliable across the unique distribution of genotypes (1, 3, and 6 being roughly equally distributed) in this cohort.

In this study, the core Ag assay was precise across most of the measurable range. The mean CV ranged from 1.67 to 9.74%, which confirmed the precision of the assay across the measurable range of core Ag concentrations and up to 150 pg/ml. Precision was lower for lesser concentrations of Core Ag (Table 2, samples with 1.5 to 25 pg/ml; Table 3, low positive control; and Table 4, calibrators (containing 1.5 and 5 pg/ml). These results suggest that precision may be suboptimal at the lower end of the measurable range and that a more sensitive quantitative RNA assay should be used to accurately determine viral load in pertinent specimens.

There were some limitations of the assay, chiefly having to do with both ends of the measurable range. We detected HCV RNA in 39% of samples that were core Ag negative. This lower sensitivity may preclude widespread use of the assay for confirmation of anti-HCV-positive ELISA results unless core Ag-negative results are routinely followed by testing for HCV RNA. In most cases, the discrepancy seemed to be due to low concentrations of viral RNA. In addition, the majority of specimens had core Ag concentrations above the measurable range of the assay. We therefore suggest that when anti-HCV-positive specimens are tested, a 10-fold dilution be assayed first, followed by testing of an undiluted aliquot of any specimen that yields a negative result.

Because it has been reported that HCV RNA levels are higher in HIV-coinfected individuals (24, 25), we hypothesized that analogous results would be found in core Ag levels. In fact, we found that median core Ag concentrations were significantly higher (2.5-fold) in coinfected subjects. We must stress, however, that our analysis included Core Ag values that were outside the measurable range of the assay and therefore not accurately quantified.

Several potential uses of the trak-C assay in monitoring HCV infection have recently been described (3, 11, 12, 28, 31). Because of the low technical requirements and high throughput of this assay compared to those of RNA assays, we believe its performance is promising for monitoring HCV levels during active infection in a clinical or research mode.

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