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Prospective Multicenter Clinical Evaluation of AMPLICOR and COBAS AMPLICOR Hepatitis C Virus Tests

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We conducted a multicenter clinical evaluation of the second versions of the manual AMPLICOR and the semiautomated COBAS AMPLICOR tests for hepatitis C virus (HCV) RNA (Roche Molecular Systems, Inc., Pleasanton, Calif.). The performance characteristics of these HCV RNA tests for diagnosis of active viral infection were determined by comparison to anti-HCV serological test results, alanine aminotransferase levels, and liver biopsy histology results. A total of 878 patients with clinical or biochemical evidence of liver disease were enrolled at four hepatology clinics. A total of 1,089 specimens (901 serum and 188 plasma) were tested with the AMPLICOR test. Sensitivity compared to serology was 93.1% for serum and 90.6% for plasma. The specificity was 97% for serum and 93.1% for plasma. A total of 1,084 specimens (896 serum and 188 plasma) were tested with the COBAS test. Sensitivities for serum and plasma were the same as with the AMPLICOR test. The specificity was 97.8% for serum and 96.6% for plasma. Of the 69 specimens with false-positive and false-negative AMPLICOR test results relative to those of serology, alternative primer set (APS) reverse transcription (RT)-PCR analysis showed that the AMPLICOR test provided the correct result relative to the specimens containing HCV RNA in 64 (92.7%) specimens. Similarly, 66 of 67 (98.5%) false-positive and false-negative COBAS test results were determined to be correct by APS RT-PCR analysis. There were no substantive differences in clinical performances between study sites, patient groups, specimen types, storage conditions (−20 to −80°C versus 2 to 8°C), or anticoagulants (EDTA versus acid citrate dextrose) for either test. Both tests showed >99% reproducibility within runs, within sites, and overall. We conclude that these tests can reliably detect the presence of HCV RNA, as evidence of active infection, in patients with clinical or biochemical evidence of liver disease.

Detection of hepatitis C virus (HCV) RNA in serum or plasma by nucleic acid amplification methods is important in confirming the diagnosis of hepatitis C, distinguishing active from resolved infection, and assessing the response to therapy. Many different in-house and commercially developed assays, based on a variety of nucleic acid amplification strategies, have been described. Nucleic acid amplification assays are part of the algorithms for diagnosis of hepatitis C proposed by the National Institutes of Health (15), the Centers for Disease Control and Prevention (3), the European Association for the Study of the Liver (7), and the National Academy of Clinical Biochemistry (6).

Assays for detection of HCV RNA based on reverse transcription (RT)-PCR are commonly used in clinical practice. Clinical laboratories participating in the College of American Pathologists nucleic acid amplification proficiency-testing program use commercially developed assays almost exclusively. In a recent survey of participants in this program (CAP 1999 ID-C), the majority (71%) performed RT-PCR with AMPLICOR HCV kits (Roche Molecular Systems, Pleasanton, Calif.).

The AMPLICOR HCV tests have been developed in a manual, microwell plate format and an automated format that uses the COBAS AMPLICOR analyzer. The first generations of both the manual and automated tests were recently modified to improve sensitivity and reliability. The modifications include a 10-fold increase in effective sample volume, an internal control molecule that is added to the specimen prior to nucleic acid extraction, and addition of a cosolvent to the reaction mixture to eliminate secondary structure in the target RNA.

Despite the widespread use of the second-generation AMPLICOR HCV tests in clinical laboratories, there is only a single published clinical evaluation (5). In this study, 187 clinical specimens and dilution panels of different HCV genotypes were tested with the first and second versions of the COBAS AMPLICOR HCV test. The second version of the test was found to have greater sensitivity and less genotype bias than the first version. In a recent study of the analytical performance characteristics of the second-generation AMPLICOR tests, the limit of detection was found to be 50 IU of HCV genotype 1 RNA/ml and all genotypes were amplified with similar efficiency (13).

Here we report the results of a multicenter clinical evalua-

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TABLE 1. Summary of patient information

Parameter	Category	Value
Age	Mean (SD)	46.2 (10.8)
	Range	10–81
Sex [no. (%)]	Males	478 (54.4)
	Females	400 (45.6)
Race [no. (%)]	Caucasian	630 (71.8)
	Black	117 (13.3)
	Hispanic	93 (10.6)
	Asian	18 (2.0)
	Native-American	2 (0.2)
	Unknown	8 (0.9)
	Other	10 (1.1)
ALT level	Mean (SD)	95.3 (79.8)
	Range	7–668
Reason for visit [no. (%)]	Prior HCV diagnosis	346 (39.4)
	HCV evaluation	302 (34.4)
	Liver disease evaluation	237 (27.0)
Prior HCV therapy [no. (%)]	Yes	160 (18.2)
	No	718 (81.8)
Clinical diagnosis [no. (%)]	Chronic HCV infection	636 (72.4)
	Autoimmune hepatitis	20 (2.3)
	Alcoholic liver disease	49 (5.6)
	Chronic HBV infection	37 (4.2)
	Primary biliary cirrhosis	45 (5.1)
	Cryptogenic cirrhosis	18 (2.0)
	Chronic renal failure	3 (0.3)
	Other	106 (12.1)
Liver biopsy performed [no. (%)]	Yes	515 (58.6)
	No	363 (41.4)

tion of the second versions of both the manual AMPLICOR and the semiautomated COBAS AMPLICOR tests for HCV RNA. These tests were recently granted marketing approval by the U.S. Food and Drug Administration to directly detect the presence of HCV in the blood. The results of these qualitative HCV RNA tests were evaluated against anti-HCV serology results, alanine aminotransferase (ALT) levels, and liver biopsy histology results. The objectives of this study were to determine the reliability and reproducibility, define the performance characteristics, and evaluate the clinical utility of these tests for patients presenting to hepatology clinics with biochemical or clinical evidence of liver disease.

MATERIALS AND METHODS

Patients. A total of 878 patients were enrolled in the study at the four sites, 137 at Emory University (EU), 344 at Virginia Commonwealth University (VCU), 232 at University of Miami (UM), and 165 at University of Washington (UW). All patients included in the study presented to hepatology clinics with biochemical or clinical evidence of liver disease, had not received HCV therapy for at least 6 months prior to enrollment, and had given written informed consent to participate in the study. The appropriate review committee at each study site approved the study protocol. A case report form was used to record patient demographic information, the reason for clinic visit, clinical diagnosis, prior HCV therapy (if previously treated), and liver biopsy results (if performed). In cases where liver biopsy was performed prior to enrollment, copies of the histology reports were obtained. The intervals between biopsy and collection of the serum or plasma for HCV RNA testing varied, so histological findings may not represent disease activity at the time the specimens were collected. The populations of patients for whom the AMPLICOR and COBAS AMPLICOR tests were evaluated differed by only four patients. The demographic information and clinical history for all of the study patients are summarized in Table 1.

The performance of the HCV RNA testing relative to that of serology testing was evaluated in all patients and, separately, in three distinct patient groups by disease state. The diagnosis group included patients who had never been treated for HCV infection and who had not undergone liver transplantation. These patients were referred to the clinics for evaluation of HCV infection or other liver diseases. The previously treated group included patients who had received antiviral therapy more than 6 months prior to enrollment in the study. Patients who had undergone liver transplantation prior to enrollment were included in the posttransplant group. ALT levels were defined as normal or elevated based on the normal ranges used at the testing sites. Histology reports on liver biopsies were categorized as having evidence of hepatitis or not having evidence of hepatitis. Histological features of hepatitis included inflammation and necrosis typical of hepatitis, and in many cases there were fibrosis and cirrhosis. Hepatitis cases included viral, autoimmune, and nonalcoholic steatohepatitis.

Specimens. Both serum and plasma specimens were collected for HCV RNA testing. Blood for serum specimens was collected in VACUTAINER serum separator tubes (Becton Dickinson, Franklin Lakes, N.J.) and for plasma specimens in either EDTA- or ACD-containing tubes (Becton-Dickinson). Whole blood was stored at 2 to 25°C for up to 6 h prior to separation into either serum or plasma. Each serum or plasma specimen was divided into a minimum of six, 220-μl single use aliquots. A number of patients had specimens prepared as both serum and plasma and had serum specimens stored both refrigerated and frozen prior to testing (Table 2). The refrigerated specimens were tested within 72 h of specimen collection. Specimens for the reference tests were collected, transported, and processed according to the standard operating procedures at the study sites.

Reference methods. Enzyme-linked immunoassays (EIA) for HCV antibodies were performed at each study site according to the manufacturers' instructions. A second-generation test (Abbott, Abbott Park, Ill.) was used at three sites (EU, VCU, and UW), and a third-generation test (Ortho, Raritan, N.J.) was used at the remaining site (UM). A second-generation recombinant immunoblot assay (RIBA) (Chiron, Emeryville, Calif.) was performed on each EIA-reactive specimen according to the manufacturer's instructions. ALT levels were determined at each site by standard methods.

Alternative primer set RT-PCR. Nested RT-PCR testing using three alternative primer sets (APS) derived from the 5′ untranslated region (UTR), core, and envelope 1 regions of the HCV viral genome was performed at UW on those specimens identified as discrepant at each site. HCV RNA was extracted from patients' specimens using Qiaprep viral RNA columns (Qiagen, Valencia, Calif.). Complementary DNA was synthesized using random hexamers and Moloney murine leukemia virus reverse transcriptase. The oligonucleotide primer sequences and methods for cDNA amplification and amplicon detection have been previously described (1, 2, 10, 11, 17).

AMPLICOR HCV Test v2.0. The AMPLICOR HCV Test version 2.0 (v2.0) is an RT-PCR in a manual, microwell format that amplifies a 244-nucleotide segment of the 5′ UTR of the HCV genome. The test was performed at all sites according to the manufacturer's instructions as previously described (13). HCV RNA optical density (OD) values were interpreted as follows: <0.3, negative; ≥ 0.3 and <1.0, equivocal; and ≥ 1.0, positive. An OD value of 0.3 was used as the cutoff for the internal control (IC) RNA. Any specimens with OD values of <0.3 for both HCV and IC wells were considered potentially inhibitory (PI).

COBAS AMPLICOR HCV Test v2.0. The COBAS AMPLICOR HCV Test v2.0 was performed at all sites according to the manufacturer's instructions as previously described (13). The COBAS AMPLICOR analyzer combines four instruments (thermal cycler, incubator, wash station, and photometer) into one system. Specimen and IC control preparation, RT, and target and IC amplification processes were essentially the same as with the manual AMPLICOR test.

TABLE 2. Number, types, and storage conditions of specimens

Site	No. of specimens				
	Serum stored at:		Plasma[a] with:		Total
	−20 to −80°C	(2 to 8°C)	EDTA	ACD	
EU	102	35	0	0	137
VCU	344	0	0	0	344
UM	232	35	35	0	302
UW	160	0	126	27	313
Total	838	70	161	27	1,096

[a] All plasma specimens were stored between −20 and −80°C.

TABLE 3. Interpretation of AMPLICOR and COBAS AMPLICOR HCV test, v2.0 results compared to HCV serology results

AMPLICOR result	EIA result	RIBA result	AMPLICOR interpretation
Positive	Positive	Positive	True positive
Positive	Positive	Indeterminate	True positive
Positive	Positive	Negative	False positive
Positive	Negative	Not done	False positive
Negative	Negative	Not done	True negative
Negative	Positive	Positive	False negative
Negative	Positive	Indeterminate	False negative
Negative	Positive	Negative	True negative

However, the test formats differ in the ways in which the amplicons are captured and detected. In the COBAS test, biotinylated amplicons are captured by magnetic particles coated with oligonucleotide probes specific for either HCV or the IC. HCV OD values were interpreted as follows: <0.15, negative; ≥ 0.15 and <1.0, equivocal; and ≥ 1.0, positive. An OD value of 0.15 was used as the cutoff for the IC. Any specimens with OD values of <0.15 for both HCV and IC wells were considered PI. The differences in the cutoff values between the COBAS and AMPLICOR HCV tests are due to the differences in the wavelengths of light used to measure the color development (660 versus 450 nm).

Clinical specimen protocol. Each investigation site collected either serum or plasma or both from patients who met the study entrance criteria. AMPLICOR HCV Test v2.0, COBAS AMPLICOR HCV Test v2.0, EIA, and ALT tests were performed on each specimen. RIBA testing was performed on all EIA-positive specimens.

Specimens that tested equivocal in either AMPLICOR test were also retested in duplicate using a second aliquot. Specimens with at least one repeat test result greater than or equal to the assay cutoff for negative were considered positive for HCV RNA. Specimens with both duplicate repeat test results less than the assay cutoff for negative were considered negative for HCV RNA provided that the IC results for both replicates were valid.

For specimens that were found to be PI in either AMPLICOR test, another aliquot of the original specimen was processed and amplified in duplicate. HCV RNA results for these samples were interpreted as described for the equivocal samples if valid IC OD values were obtained. If no valid IC OD value was obtained on retesting, the sample remained PI.

The criteria used to interpret the AMPLICOR test results relative to serology results are shown in Table 3. The AMPLICOR test results obtained in each test format were evaluated against the serological results independently. APS RT-PCR analysis was performed on all specimens with false-positive and false-negative AMPLICOR test results relative to serology results.

Reproducibility study. A reproducibility study was performed to assess the within-day, within-site, and total reproducibilities of the AMPLICOR and the COBAS AMPLICOR HCV tests. Qualified testing personnel at three study sites (EU, UM, and UW) and at Roche Molecular Systems participated in the study. A commercially available panel of specimens was used (BBI Panel MHW402; Boston, Mass.). Each plasma panel contained eight tubes consisting of four negative and four positive samples containing 200, 400, 500, and 50,000 HCV copies/ml. The panel samples and the kit controls were tested in duplicate on each day for 4 days at one site (UM) and for 5 days at the other sites. The panel samples were coded, and the participants were blinded to the expected results.

Data analysis. The parameters use to assess the performance characteristics of the tests relative to serology were prevalence, sensitivity, and specificity. Four separate analyses were performed using these three parameters by study site and across all sites. Agreement with EIA results were assessed separately in the diagnosis, previously treated, and posttransplant patient groups, and correlated with the ALT levels in liver histology reports.

Reproducibility was assessed by computing the proportion of true positives and true negatives, separately, by run number and concentration of HCV RNA copies/ml in each sample by site. Sites were combined, and the same proportions were calculated across sites by concentration of HCV RNA.

Statistical software SAS version 6.12 was used to analyze the data and compute the 95% confidence intervals. All 95% confidence intervals are exact intervals calculated using cumulative binomial methods.

RESULTS

The clinical performance of the AMPLICOR HCV Test v2.0 versus that of serology across all sites and patient groups is shown in Table 4. Overall, 905 serum and 188 plasma specimens were tested at the four sites with the AMPLICOR Test. There were three unresolved PI serum specimens and one unresolved equivocal serum specimen that were excluded from the data analysis. Therefore, valid test results were available for 901 (99.6%) serum specimens. Valid AMPLICOR results were available for all of the plasma samples tested. The prevalence of HCV antibody was 69.4% in the serum group and 76.6% in the plasma group. The AMPLICOR test with serum samples had a sensitivity of 93.1% and a specificity of 97% compared to serology. The test performance characteristics with plasma samples were similar (sensitivity, 90.6%; specificity, 93.1%).

There were no substantive differences in test performance characteristics between the study sites (Table 4). In addition, there were no notable differences in clinical performance between serum samples stored at 2 to 8°C versus −20 to −80°C and EDTA-plasma versus ACD-plasma (data not shown).

There were 46 serum samples classified as false negative when compared with serology. Forty-five samples were retested, and 44 (97.8%) were found to be negative for HCV RNA in all three APS RT-PCR assays. All of the 15 false-negative plasma specimens were negative for HCV RNA in all of the APS RT-PCR assays. False-positive AMPLICOR results occurred with seven serum and two plasma specimens. Four of

TABLE 4. Clinical performance of AMPLICOR HCV Test v2.0

Site(s)	Specimen type	Total no. of specimens	No. of specimens with indicated result[a]				% Prevalence	Versus HCV serology	
			TP	TN	FP	FN		% Sensitivity (95% CI)[b]	% Specificity (95% CI)
EU	Serum	136	113	13	0	10	83.1	91.9 (85.6–96.0)	100 (73.5–100)
VCU	Serum	343	212	117	3	11	61.8	95.1 (91.3–97.5)	97.5 (92.9–99.5)
UM	Serum	266	173	77	3	13	65.0	93.0 (88.3–96.2)	97.5 (89.4–99.2)
UM	Plasma	35	25	8	0	2	71.4	92.6 (75.7–99.1)	100 (63.1–100)
UW	Serum	156	124	19	1	12	79.5	91.2 (85.1–95.4)	95.0 (75.1–99.9)
UW	Plasma	153	119	19	2	13	77.8	90.2 (83.7–94.7)	95.2 (69.6–98.8)
All	Serum	901	622	226	7	46	69.4	93.1 (90.9–94.9)	97.0 (93.9–98.8)
	Plasma	188	144	27	2	15	76.6	90.6 (84.9–94.6)	93.1 (77.2–99.2)

[a] TP, true positive; TN, true negative; FP, false positive; FN, false negative in comparison with HCV serology results as defined in Table 3.
[b] CI, confidence interval.

TABLE 5. Clinical performance of the COBAS AMPLICOR HCV Test v2.0

Site(s)	Specimen type	Total no. of specimens	No. of specimens with indicated result[a]				% Prevalence	Versus HCV serology	
			TP	TN	FP	FN		% Sensitivity (95% CI)[b]	% Specificity (95% CI)
EU	Serum	137	114	13	0	10	83.2	91.9 (85.7–96.1)	100 (75.3–100)
VCU	Serum	341	213	114	3	11	62.5	95.1 (91.4–97.5)	97.4 (92.7–99.5)
UM	Serum	265	173	77	2	13	65.3	93.0 (88.3–96.2)	97.5 (91.2–99.7)
UM	Plasma	35	25	8	0	2	71.4	92.6 (75.7–99.1)	100 (63.1–100)
UW	Serum	153	122	19	0	12	79.7	91.0 (84.9–95.3)	100 (82.4–100)
UW	Plasma	153	119	20	1	13	77.8	90.2 (83.7–94.7)	95.2 (76.2–99.9)
All	Serum	896	622	223	5	46	69.4	93.1 (90.9–94.9)	97.8 (95.0–99.3)
	Plasma	188	144	28	1	15	76.6	90.6 (84.9–94.6)	96.6 (82.2–99.9)

[a] TP, true positive; TN, true negative; FP, false positive; FN, false negative in comparison with HCV serology results as defined in Table 3.
[b] CI, confidence interval.

the serum and one of the plasma specimens were positive for HCV RNA in at least one of the APS RT-PCR assays. Thus, of the 69 samples classified as either false negative or false positive relative to serology, the AMPLICOR test provided the correct result relative to the specimen containing HCV RNA in 64 (92.7%) specimens.

Only five (0.6%) serum and no plasma specimens had OD values that were in the equivocal range (0.3 to 1) of the AMPLICOR assay. Four of the five sera were retested in duplicate. Upon retesting, two resolved as positive and two resolved as a negative.

Ten (1.1%) serum specimens were found to be PI on initial testing. Eight of these were retested and only one remained PI. Of the seven specimens that gave valid IC results on retesting, six were HCV RNA negative and one was HCV RNA positive. Four (2.1%) plasma samples were found to be PI on initial testing. All gave valid IC results on retesting (three negative and one positive for HCV RNA).

The clinical performance of the COBAS test versus that of serology across all sites and patient groups is shown in Table 5. Overall, 908 serum and 188 plasma specimens were tested at the four sites. All but 12 (1.3%) serum specimens gave valid COBAS test results, including eight unresolved PI and four unresolved equivocal specimens. These specimens were excluded from the data analysis. Valid COBAS test results were available for all plasma specimens. The prevalence of HCV antibody was 69.4% in the serum group and 76.6% in the plasma group. The COBAS test with serum specimens had a sensitivity of 93.1% and a specificity of 97.8% compared to serology. The test performance characteristics with plasma specimens were similar (sensitivity, 90.6%; specificity, 96.6%).

There were no substantive differences in test performance characteristics between the study sites (Table 5). In addition, no notable differences in clinical performance between serum specimens stored at 2 to 8°C versus −20 to −80°C and between EDTA-plasma versus ACD-plasma were found (data not shown).

There were 46 serum and 15 plasma specimens that were HCV antibody positive but negative for HCV RNA in the COBAS test. All of these samples were also negative for HCV RNA in all three alternative primer pair RT-PCR assays. False-positive COBAS test results occurred with five serum and one plasma specimens. Four of the sera and the single plasma specimen were positive in at least one of the APS RT-PCR assays. Thus, of the 67 samples with discrepant antibody and RNA results, the COBAS test provided the correct result relative to the sample containing HCV RNA in 66 (98.5%) samples.

Only 12 (1.3%) sera and one (0.5%) plasma specimen had OD values that were in the equivocal range in the COBAS test. Eight of the 12 sera and the single plasma specimen were retested in duplicate. Upon retesting, one serum and the plasma specimen resolved as positive, and seven serum specimens resolved as negative. The seven resolved HCV RNA-negative sera were also negative in all three of the APS RT-PCR assays.

Twenty-seven (3%) serum and no plasma specimens were found to be PI on initial testing with the COBAS test. Twenty-four of the sera were retested, and only five remained PI. Of the 19 specimens that gave valid IC results upon retesting, 3 were positive and 16 were negative for HCV RNA.

Both AMPLICOR and COBAS test results were available for 1,076 specimens. The results were concordant for 1,071 (99.5%) specimens. The discordant test results for the five

TABLE 6. Summary of results for specimens that were discordant in the AMPLICOR HCV v2.0 tests

Site	Specimen no.	Specimen type	AMPLICOR	COBAS AMPLICOR	EIA	RIBA	APS RT-PCR
VCU	254	Serum (frozen)	Negative	Positive	Positive	Positive	Positive[a]
UM	62	Serum (frozen)	Positive	Negative	Positive	Positive	Negative
UM	209	Serum (frozen)	Positive	Negative	Negative	ND[b]	Negative
UW	35	Serum (frozen)	Positive	Negative	Negative	ND	Positive[c]
UW	1157	Plasma (ACD)	Positive	Negative	Negative	ND	Negative

[a] Positive in 5′ UTR, core, and E1 APS RT-PCR.
[b] ND, not determined.
[c] Positive only in the E1 APS RT-PCR.

TABLE 7. Performance of the COBAS AMPLICOR HCV Test v2.0 compared to serological, biochemical, and histological findings in the diagnosis patient group

Histological findings	Serology results	No. of PCR-positive cases	No. of PCR-negative cases	% Proportion agreeing with EIA results (95% CI)[a]
Elevated ALT and hepatitis	EIA+/RIBA+	162	2	98.8 (95.7–99.8)
	EIA+/RIBA IND	2	0	100.0 (15.8–100.0)
	EIA+/RIBA−	0	0	
	EIA−	0	16	100.0 (79.4–100.0)
Normal ALT and hepatitis	EIA+/RIBA+	58	0	100.0 (93.8–100.0)
	EIA+/RIBA IND	1	0	100.0 (2.5–100.0)
	EIA+/RIBA−	0	0	
	EIA−	0	9	100.0 (66.4–100.0)
Elevated ALT and no hepatitis	EIA+/RIBA+	1	0	100.0 (2.5–100.0)
	EIA+/RIBA IND	0	0	
	EIA+/RIBA−	0	0	
	EIA−	0	19	100.0 (82.4–100.0)
Normal ALT and no hepatitis	EIA+/RIBA+	0	1	0.0 (0.0–97.5)
	EIA+/RIBA IND	1	0	100.0 (2.5–100.0)
	EIA+/RIBA−	0	0	
	EIA−	1	21	95.5 (77.2–99.9)

[a] CI, confidence interval.

TABLE 8. Performance of the COBAS AMPLICOR HCV Test v2.0 compared to serological, biochemical, and histological findings in the previously treated patient group

Histological findings[a]	Serology results	No. of PCR-positive cases	No. of PCR-negative cases	% Proportion agreeing with EIA result (95% CI)[b]
Elevated ALT and hepatitis	EIA+/RIBA+	85	2	97.7 (91.9–99.7)
	EIA+/RIBA IND	4	0	100.0 (2.5–100.0)
	EIA+/RIBA−	0	0	
	EIA−	0	0	
Normal ALT and hepatitis	EIA+/RIBA+	22	5	81.5 (61.9–93.7)
	EIA+/RIBA IND	0	1	0.0 (0.0–97.5)
	EIA+/RIBA−	0	0	
	EIA−	0	0	
Elevated ALT and no hepatitis	EIA+/RIBA+	0	1	0.0 (0.0–97.5)
	EIA+/RIBA IND	0	0	
	EIA+/RIBA−	0	0	
	EIA−	0	0	

[a] There were no cases showing normal ALT levels and no hepatitis in this patient group.
[b] CI, confidence interval.

remaining specimens are shown in Table 6. APS RT-PCR analysis confirmed the COBAS test result in four of five cases.

We also compared the serological and COBAS test results separately in the diagnosis, previously treated, and posttransplant patient groups. Only serum specimens and patients with liver biopsy results were included in this analysis. In each patient group we assessed the agreement between the EIA and COBAS test results by disease state as determined by ALT levels and liver biopsy results.

The overall agreement between EIA and COBAS test results was 98.6% for the 294 patients included in the diagnosis group (Table 7). Among patients with elevated ALT levels and hepatitis as determined by histology, HCV RNA was detected in 164 of 166 (98.8%) EIA-positive and in none of the 16 EIA-negative patients. HCV RNA was detected in all of the 59 EIA-positive and none of the 9 EIA-negative patients with normal ALT levels and hepatitis. Complete concordance between the EIA and COBAS test results was found in the 20 patients with elevated ALT and no hepatitis. The single EIA-positive patient in this category also had HCV RNA detected in serum. In patients with normal ALT levels and no hepatitis, there was 91.7% agreement between the EIA and COBAS test results. HCV RNA was detected in one of two (50%) EIA-positive patients and in one of 22 (4.5%) EIA-negative patients in this category.

The overall agreement between EIA and COBAS test results was 92.5% for the 120 patients included in the previously treated group (Table 8). All of the patients in this group were EIA positive, and all but one had hepatitis. Among patients with hepatitis, HCV RNA was detected in 89 of 91 (97.8%) patients with elevated ALT levels and in 22 of 28 patients (78.6%) with normal ALT levels. No HCV RNA was detected in the remaining patient in this group, who had an elevated ALT level but no evidence of hepatitis on biopsy.

The overall agreement between EIA and COBAS test results was 87.5% for the 16 patients who had undergone liver transplantation (Table 9). Fourteen patients were EIA positive. HCV RNA was not detected in two EIA-positive patients, one with normal ALT levels and hepatitis, and the other with normal ALT levels and no hepatitis.

The AMPLICOR test results in the three patient groups were very similar to those obtained with the COBAS test (data not shown). There were too few plasma specimens to make meaningful comparisons with the other markers of liver disease in the different patient groups.

During the study there were 101 runs of the AMPLICOR test, of which 93 (92%) provided valid results. Of the eight failed runs, five (62.5%) were due to positive controls being low, one (12.5%) was due to both a positive and negative control out of range, and two (25%) runs were repeated because the samples used in the run were past the storage stability date.

TABLE 9. Performance of the COBAS AMPLICOR HCV Test v2.0 compared to serological, biochemical, and histological findings in the posttransplant patient group

Histological findings[a]	Serology results	No. of PCR-positive cases	No. of PCR-negative cases	% Proportion agreeing with EIA results (95% CI)[b]
Elevated ALT and hepatitis	EIA+/RIBA+	9	0	100.0 (66.3–100.0)
	EIA+/RIBA IND	1	0	100.0 (2.5–100.0)
	EIA+/RIBA−	0	0	
	EIA−	0	0	
Normal ALT and hepatitis	EIA+/RIBA+	2	0	100.0 (15.8–100.0)
	EIA+/RIBA IND	0	1	0.0 (0.0–97.5)
	EIA+/RIBA−	0	0	
	EIA−	0	0	
Normal ALT and no hepatitis	EIA+/RIBA+	0	1	0.0 (0.0–97.5)
	EIA+/RIBA IND	0	0	
	EIA+/RIBA−	0	0	
	EIA−	0	2	100.0 (15.8–100.0)

[a] There were no cases showing elevated ALT levels and no hepatitis in this patient group.
[b] CI, confidence interval.

TABLE 10. Reproducibility of the AMPLICOR and COBAS AMPLICOR HCV v2.0 tests[a]

No. of HCV RNA copies/ml	Correct result	AMPLICOR			COBAS AMPLICOR		
		No. of specimens tested	% Correct result	95% CI[b]	No. of specimens tested	% Correct result	95% CI
0	Negative	153	99.3	96.4–99.9	171	100	97.9–100
200	Positive	38	100	90.1–100	43	100	91.8–100
300	Positive	38	100	90.8–100	42	100	91.6–100
500	Positive	38	97.4	86.2–99.9	43	97.7	87.1–99.9
50,000	Positive	38	100	90.8–100	43	100	91.8–100

[a] Pooled data from four test sites (EU, UM, UW, and Roche Molecular Systems). Results for five PI panel members were excluded from the data analysis (one in AMPLICOR and four in COBAS AMPLICOR).
[b] CI, confidence interval.

There were 394 runs of the COBAS HCV attempted during the study, of which 357 (91%) provided valid results. Of the 37 runs of study specimens which failed, 6 (16%) were due to positive controls being low, 5 (14%) were due to the negative control out of range, and 3 (8%) were due to both a positive and negative control out of range. Also, 18 (48%) runs were repeated because the samples used in the run were past the storage stability date. Finally, mechanical problems with the analyzer resulted in five (14%) failed runs.

The reproducibility study results across all sites for the AMPLICOR and the COBAS tests are summarized in Table 10. Examination of the AMPLICOR test results shows that of 153 negative panel members tested only 1 gave a positive result (99.3% correct results). Of the 151 positive panel members tested, only 1, at a concentration of 500 copies/ml, was falsely negative (99.3% correct results). One negative panel member was PI and dropped from the analysis.

Examination of the COBAS test results shows that of 171 negative panel members tested, all samples were correctly identified as negative (100% correct results). Of the 171 positive panel members tested, only 1 panel member, at a concentration of 500 copies/ml, was falsely negative (99.4% correct results). Four panel members were PI and not included in the data analysis.

Reproducibility within runs and within sites was also >99% for both tests (data not shown). All sites were able to detect all the panel members with the lowest concentration of HCV RNA (200 copies/ml). Therefore, the analytical sensitivity for both tests, as assessed with this panel, was ≤200 copies/ml.

DISCUSSION

Our study is the first multicenter clinical evaluation of the AMPLICOR and COBAS AMPLICOR HCV v2.0 tests. The patients in this study were enrolled at four liver disease clinics in the United States and had a high prevalence of HCV infection. The two tests were evaluated independently using samples obtained from the same patients and were found to have almost identical test performance characteristics when serology was used as the diagnostic "gold standard". The results for the AMPLICOR and COBAS tests were also found to be highly concordant with each other.

We found that approximately 7% of HCV antibody-positive specimens had no detectable RNA by the AMPLICOR methods. These were scored as false-negative AMPLICOR results. However, all but one specimen also had no detectable HCV RNA by the APS RT-PCR used in the discrepant sample analysis. We conclude that the RNA-negative patients were not viremic at the time the samples were drawn due to either intermittent viremia or viral clearance. Our study could not distinguish between these two possibilities since patients were sampled at only one time point.

We demonstrated that four test sites could consistently detect samples containing as few as 200 HCV RNA copies/ml. The manufacturer has shown that the second-generation tests can detect 100% of samples containing as little as 50 IU/ml (approximately 100 copies/ml) (13). Although the fluctuations in viral load that occur in patients chronically infected with HCV are not well defined, it is rare to find patients with viral loads of $<10^4$ copies/ml in the absence of therapy (8, 12, 14, 15, 17–20). Thus, it appears that the analytical sensitivity of the AMPLICOR tests is at least 100-fold lower than the lowest value likely to be encountered in patients being evaluated for HCV infection prior to therapy. However, enhanced sensitivity may be required for other applications such as detection of HCV RNA in blood donor plasma pools (9) or assessment of virologic response at the end of treatment (16). Recently, a transcription-mediated amplification assay (Gen-Probe, San Diego, Calif.) with a sensitivity of 10 to 50 copies/ml was shown to detect HCV RNA in 36% of patients who were classified as virologic end-of-treatment responders with subsequent relapse according to the results of the AMPLICOR HCV Test v2.0 (16).

The inclusion of an IC that is extracted and amplified simultaneously with the clinical specimen increases the level of confidence in negative RT-PCR results. However, only 1.1 and 3% of specimens tested with AMPLICOR and COBAS tests, respectively, had invalid IC results. Most (75%) of the IC failures were probably due to inefficient nucleic acid extraction rather than to the presence of inhibitors in the specimens because the problem resolved upon retesting. Recovery of the RNA pellet is the most technically demanding step in these procedures. Although no standards exist for an acceptable number of false-negative tests, the low rates reported here call into question the need for an IC. The inclusion of an IC adds substantial expense to the tests and, in our experience, was of limited value. A more cost-effective strategy in a diagnostic setting would be to omit the IC and simply retest all patients with a negative HCV RNA test and high index of clinical suspicion of active infection.

Both the AMPLICOR and COBAS AMPLICOR tests were very specific relative to serology (≥97%). Overall, HCV RNA was detected by APS RT-PCR analysis in five of eight (62%) clinical specimens with false-positive test results. The reason

for the negative antibody test results for the patients with confirmed viremia was not clear. Three of these patients were previously documented to be HCV antibody positive and chronically infected with HCV. Circulating immune complexes or a general state of immunosuppression may have accounted for the loss of a detectable HCV antibody response in these patients. The remaining two patients had no identified risk factors for HCV infection and other liver diseases. Either these patients were truly infected with HCV but had not seroconverted or, more likely, their specimens were mislabeled or contaminated with HCV during processing.

The AMPLICOR and COBAS tests have several features that may account for the low false-positive rates observed in this study. Uracil-*N*-glycosylase and dUTP are incorporated into the reaction mixtures to avoid false positives due to product carryover from one sample to another (14). The establishment of equivocal zones for the test results and an algorithm for retesting equivocal samples also reduced the number of false-positive results. However, these features cannot prevent false-positive tests that may result from cross contamination of specimens with target nucleic acid during processing.

We also evaluated the clinical performances of the AMPLICOR and COBAS tests, independently, for patients presenting for diagnosis or evaluation of liver disease, for patients who had received antiviral therapy for hepatitis C more than 6 months prior to enrollment, and for patients who had undergone liver transplantation. Overall, we found a high concordance between the RNA and serological test results in all three patient groups, which was independent of disease state. The results were concordant in 98.6, 92.5, and 87.5% of patients in the diagnosis, previously treated, and posttransplant groups, respectively. The lower overall concordance between the RNA and serological test results in the previously treated and posttransplant groups probably is the result of the clinical interventions rather than any problems with the RNA tests in these groups of patients.

Serious concerns about HCV RNA testing have been raised by the poor performance of laboratories that participated in an HCV RNA proficiency-testing program (4). In this performance survey of 86 laboratories performing HCV RNA detection, only 16% of the laboratories performed faultlessly. Survey participants using the earlier versions of the AMPLICOR tests tended to have a sufficient quality score more often than those labs using in-house-developed methods (64 versus 45%; $P = 0.11$). We found both the AMPLICOR and COBAS v.2.0 tests to be highly reproducible at four different test sites. All sites reported >99% correct results for a total of 324 negative and 323 positive panel members. In addition, there were no substantive differences between the sites in the test performance characteristics with clinical specimens.

Both the manual and automated formats for the HCV RNA tests were highly reliable, but, surprisingly, the COBAS AMPLICOR analyzer did not improve the reliability or reproducibility of the HCV RNA test. However, the labor savings in laboratories doing large numbers of tests may offset the expense of the instrument.

Both HCV RNA tests could be performed using serum, EDTA-plasma, or ACD-plasma with no substantive differences in test performance characteristics. We also found no differences in test performance characteristics attributable to the temperatures at which the specimens were stored prior to testing (refrigerated versus frozen).

Our data support the current recommendation that a positive HCV EIA test should be confirmed by a qualitative test for HCV RNA rather than by RIBA in patients with a high pretest probability of infection (6, 7). The RIBA was negative in only 1 (0.1%) and was indeterminate in 34 (4.2%) EIA-positive patients. The AMPLICOR HCV tests detected the presence of HCV RNA in 62% of the RIBA-indeterminate specimens and, thus, confirmed that these patients were actively infected with HCV. HCV RNA tests can distinguish active from resolved infections; however, a single negative RNA test is not conclusive evidence of resolved infection because viremia in chronically infected individuals may be intermittent (8, 11). In our population only 7% of EIA-positive patients were RNA negative and would have required additional testing to establish whether their infection was active or resolved.

In conclusion, both the AMPLICOR and COBAS AMPLICOR v2.0 HCV tests demonstrated good overall agreement with serology results and other markers of liver disease and had excellent reproducibility across study sites and specimen types. These tests can reliably detect the presence of HCV RNA, as evidence of active infection, in patients with clinical or biochemical evidence of liver disease. The performance characteristics of these tests in low-prevalence patient populations or as a means to assess end-of-treatment responses are yet to be established.

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REFERENCES

1. **Bukh, J., R. H. Purcell, and R. H. Miller.** 1992. Importance of primer selection for the detection of hepatitis C virus RNA with the polymerase chain reaction assay. Proc. Natl. Acad. Sci. USA **89:**187–191.
2. **Bukh, J., R. H. Purcell, and R. H. Miller.** 1994. Sequence analysis of the core gene of 14 hepatitis C virus genotypes. Proc. Natl. Acad. Sci. USA **91:**8239–8243.
3. **Centers for Disease Control and Prevention.** 1998. Recommendations for prevention and control of hepatitis C virus (HCV) infection and HCV-related chronic disease. Morb. Mortal. Wkly. Rep. **47:**1–39.
4. **Damen, M., H. T. M. Cuypers, H. L. Zaaijer, H. W. Reesink, W. P. Schaasberg, W. H. Gerlich, H. G. M. Niesters, and P. N. Lelie.** 1996. International collaborative study on the second EUROHEP HCV-RNA reference panel. J. Virol. Methods **58:**175–185.
5. **Doglio, A., C. Laffont, F. X. Caroli-Bosc, P. Rochet, and J.-C. Lefebvre.** 1999. Second generation of the automated Cobas Amplicor HCV assay improves sensitivity of hepatitis C virus RNA detection and yields results that are more clinically relevant. J. Clin. Microbiol. **37:**1567–1569.
6. **DuFour, D. R., J. A. Lott, F. S. Nolte, D. Gretch, R. S. Koff, and L. B. Seeff.** 2000. Diagnosis and monitoring of hepatic injury. II. Recommendations for use of laboratory tests in screening, diagnosis, and monitoring. Clin. Chem. **46:**2050–2068.
7. **European Association for the Study of the Liver.** 1999. EASL International Consensus Conference on Hepatitis C. Paris, 26–28, February 1999, Consensus statement. J. Hepatol. **30:**956–961.
8. **Fanning, L., E. Kenny-Walsh, J. Levis, K. R. Choudhury, B. Cannon, M. Sheehan, M. Whelton, and F. Shanahan.** 2000. Natural fluctuations of hepatitis C viral load in the homogeneous patient population: a prospective study. Hepatology **31:**225–229.
9. **Gallarda, J. L., and E. Dragon.** 2000. Blood screening by nucleic acid amplification technology: current issues, future challenges. Mol. Diagn. **5:**11–22.
10. **Gretch, D., J. J. Wilson, R. L. Carithers, Jr., C. dela Rosa, J. H. Han, and L. Corey.** 1993. Detection of hepatitis C virus RNA: comparison of one-stage polymerase chain reaction (PCR) with nested-set PCR. J. Clin. Microbiol. **31:**289–291.

11. **Gretch, D., W. Lee, and L. Corey.** 1992. Use of aminotransferase, hepatitis C antibody, and hepatitis C polymerase chain reaction RNA assays to establish the diagnosis of hepatitis C virus infection in a diagnostic virology laboratory. J. Clin. Microbiol. **30:**2145–2149.
12. **Hagiwara, H., N. Hayashi, E. Mita, M. Naito, A. Kasahara, and H. Fusamoto.** 1993. Quantitation of hepatitis C virus RNA in serum of asymptomatic blood donors and patients with type C chronic liver disease. Hepatology **17:**545–550.
13. **Lee, S. C., A. Anthony, J. Leibox, J. Q. Yang, S. Soviero, K. Gutekunst, and M. Rosensraus.** 2000. Improved version 2.0 qualitative and quantitative AMPLICOR reverse transcription-PCR tests for hepatitis C virus RNA: calibration to international units, enhanced genotype reactivity, and performance characteristics. J. Clin. Microbiol. **38:**4171–4170.
14. **Longo, M. C., M. S. Berninger, and J. L. Hartley.** 1990. Use of uracil DNA glycosylase to control carry-over contamination in polymerase chain reactions. Gene **93:**125–128.
15. **National Institutes of Health.** 1997. National Institutes of Health Consensus Development Conference Panel Statement: management of hepatitis C. Hepatology **26**(3 Suppl. 1)**:**2S–10S.
16. **Sarrazin, C., G. Teuber, R. Kokka, H. Rabenau, and S. Zeuzem.** 2000. Detection of residual hepatitis C virus RNA by transcription-mediated amplification of patients with complete virologic response according to polymerase chain reaction-based assays. Hepatology **32:**818–823.
17. **Sullivan, D. G., J. J. Wilson, R. L. Carither, J. D. Perkins, and D. Gretch.** 1998. Multigene tracking of hepatitis C virus quasispecies after liver transplantation: correlation of genetic diversification in the envelope region with asymptomatic or mild disease patterns. J. Virol. **72:**10036–10043.
18. **Trabaud, M. D., F. Bailly, S. N. Si-Ahmed, P. Chevallier, M. Sepetjan, G. Colucci, and C. Trepo.** 1997. Comparison of HCV RNA assays for the detection and quantification of hepatitis C virus RNA levels in serum of patients with chronic hepatitis C treated with interferon. J. Med. Virol. **52:**105–112.
19. **Villano, S. A., D. Vlahov, K. E. Nelson, S. Cohen, and D. L. Thomas.** 1999. Persistence of viremia and the importance of long-term follow-up after acute hepatitis C infection. Hepatology **29:**908–914.
20. **Zeuzem, S., J. H. Lee, A. Franke, B. Ruster, O. Prummer, G. Herrmann, and W. K. Roth.** 1998. Quantification of the initial decline of serum hepatitis C virus RNA and response to interferon alfa. Hepatology **27:**1149–1156.