Evaluation of the Core Antigen Assay as a Second-Line Supplemental Test for Diagnosis of Active Hepatitis C Virus Infection

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The British Columbia Center for Disease Control laboratory performs approximately 95% of all hepatitis C virus (HCV) antibody tests for the province’s 4 million inhabitants. In 2002, the laboratory tested 96,000 specimens for anti-HCV antibodies, of which 4,800 (5%) were seroreactive and required confirmation of active infection. Although HCV RNA assays with a sensitivity of 50 IU/ml or less are recommended for the confirmation of active HCV infection, given the large number of seroreactive specimens tested annually, we evaluated the Ortho trak-C assay (OTCA) as a second-line confirmatory test and determined its limit of detection (LoD).

Of 502 specimens from treatment-naive anti-HCV-positive individuals, 478 had sufficient volumes for evaluation by the OTCA and HCV RNA tests. Core antigen was not detected in 147 of 478 (30.8%) of these specimens, of which 37 of 147 (25.2%) were shown to be viremic by the VERSANT HCV (version 3.0) (branched-DNA) assay and/or the VERSANT HCV qualitative assay. Testing of 144 replicates of a World Health Organization standard dilution series indicated that the LoD of OTCA was $\approx 27,000$ IU/ml. This LoD is consistent with the inability of OTCA to detect core antigen in clinical specimens with low viral loads. We conclude that OTCA has limited value as a confirmatory test for the diagnosis of active HCV infection because 37 of 367 (10%) of viremic specimens had undetectable core antigen. Qualitative HCV RNA testing remains the present standard for the confirmation of active HCV infection in the diagnostic setting.

Accurate diagnosis of active hepatitis C virus (HCV) infection is important not only because of its associated morbidity and mortality but also because of the possibility of spontaneous or pharmacology-induced sustained virologic cure. An estimated 130 million people worldwide are infected with HCV (3, 9), of whom approximately 25% (range, 15 to 45%) will clear the infection spontaneously (1, 18) and 75% will remain chronically infected (19, 30). Among individuals who do not clear the virus and who do not receive some therapeutic intervention, 15 to 25% will progress to end-stage liver disease, may be at risk of developing hepatocellular carcinoma, or may require liver transplantation (31). In contrast, pharmacotherapy with the current combination of pegylated interferon and ribavirin can result in a sustained virologic response in about 55% of treated patients (8, 21, 23). A sustained viral response has been associated with a sustained reduction in hepatic inflammation and fibrosis and improved long-term outcomes (4, 24). These findings underscore the need for the timely and accurate identification of active HCV infection.

Unfortunately, no tests that can both screen for past infection and identify active HCV infection are available at present. Detection of anti-HCV antibodies by enzyme immunoassays (EIAs) is the screening method of choice (2); present anti-HCV EIAs are typically reactive for at least 99% of immunocompetent infected individuals (29). However, antibody responses cannot usually be detected until the first 4 to 6 weeks of infection in immunocompetent patients, and a blunted or absent antibody response may occur in immunosuppressed patients. In addition, individuals who spontaneously clear the infection may remain anti-HCV antibody positive for multiple decades or even for life (18). Moreover, even the most recent serological tests yield false-positive or indeterminate results at rates of 11% or higher (2, 14). Recombinant immunoblot assays have been used as supplemental assays to confirm anti-HCV antibody specificity but are subject to the limitations of serological testing and cannot be used to distinguish between past and active HCV infections (2, 14). Hence, at present confirmation of active HCV infection requires additional testing.

The guidelines from the National Institutes of Health recommend that qualitative HCV RNA tests with sensitivities of 50 IU/ml or less be used both to confirm the diagnosis of HCV infection and establish clearance of the virus after therapy (27). At present, qualitative HCV RNA testing is the method of choice for the confirmation of active infection in both immunocompetent patients who are anti-HCV antibody positive and immunosuppressed patients who may not mount an antibody response, as well as for assessment of both spontaneous viral clearance and viral clearance as a result of therapy. Qualitative HCV RNA tests can also detect viremia within 1 to 3 weeks of infection, which is earlier than the time of detection of seroconversion, which typically occurs within 4 to 10 weeks after the time of infection (13, 18, 28). Nevertheless, HCV RNA testing is expensive and time-consuming, requires a sophisticated molecular laboratory, and may not always be readily available in underdeveloped parts of the world, where the greatest numbers of HCV-infected patients are found (19).

Recently, an EIA for the detection and quantification of HCV core antigen has been developed that can be used as a confirmatory test for active HCV infection.
HCV core antigen, the Ortho trak-C assay (OTCA), which can be used to demonstrate active infection, has been developed by Ortho Clinical Diagnostics (Raritan, N.J.). OTCA is easy to perform, gives final results in about 3.5 h, is compatible with present EIA systems, is theoretically less prone to sample carryover than assays based on gene amplification technology, and has high degrees of specificity and reproducibility (17, 35, 37). As such, this assay provides an attractive alternative to HCV RNA testing for the confirmation of active infection. Earlier published studies have focused on the ability of OTCA to narrow the window period for the screening of blood products and its utility in the monitoring of the response to treatment (7, 12, 22, 32, 36). In the study described here, we evaluated the ability of OTCA to serve as a second-line supplemental assay for the diagnosis of active HCV infection in a general population in British Columbia. We also examined the correlation between the concentration of HCV core antigen measured by OTCA and the viral load measured by a quantitative HCV RNA test and estimated the limit of detection (LoD) of OTCA.

MATERIALS AND METHODS

Testing of patient specimens. In January 2003, 502 EDTA-anticoagulated plasma specimens from treatment-naïve individuals previously shown to be anti-HCV antibody positive were sent to the British Columbia Center for Disease Control (BCCDC) laboratory for confirmation of active infection. The anti-HCV antibody positive status was reconfirmed for 495 specimens by testing by the AxSYM HCV assay (version 3.0; Abbott Diagnostics, Chicago, Ill.). Of these, 478 specimens had sufficient volume for the comprehensive testing outlined below. All 478 specimens were tested for HCV core antigen by OTCA (Ortho Clinical Diagnostics), for which the cutoff set by the manufacturer is 1.5 pg/ml (OTCA, version 6.0; package insert; Ortho Clinical Diagnostics). According to the instructions of the manufacturer, specimens with absorbance values above the linear range for the assay were diluted 1:10 with the negative control and retested. The 478 specimens were also tested for HCV RNA by the quantitative VERSANT HCV assay (version 3.0; a branched-DNA [bDNA] assay; Bayer Healthcare LLC, Tarrytown, N.Y.), which has a limit of quantification (LoQ) of 615 IU/ml (5). The HCV bDNA assay is not intended for use for the confirmation of active infection but, rather, is intended for use in vitro by health care professionals to help manage HCV-infected patients undergoing therapy and to predict a nonsustained virologic response. However, for the purposes of this study, specimens in which the quantity of virus was within the dynamic range of the HCV bDNA assay were deemed to be viremic. All specimens yielding signals below the quantification limit of the HCV bDNA assay were tested by the VERSANT quantitative assay (HCV TMA; Bayer Healthcare LLC), which has a LoQ of about 9.6 IU/ml (10, 11). Specimens that were reactive by HCV TMA were deemed to be viremic. Selected specimens, such as those that were reactive for HCV RNA by the HCV bDNA assay or HCV TMA but that were not reactive for core antigen by OTCA were tested by the qualitative AMPLICOR HCV (version 2.0) test (Roche Molecular Systems, Pleasanton, Calif.) when sufficient sample volume was available. The HCV genotype was determined only when a request for genotyping was made by the physician and was performed by the VERSANT HCV genotyping assay (a line probe assay produced by Innogenetics, Ghent, Belgium, and distributed by Bayer Healthcare LLC). All tests were performed according to the instructions of the manufacturer.

Evaluation of sensitivity of OTCA. The HCV RNA 96/790 standard (33) prepared by the World Health Organization (WHO) was used to estimate the LoD of OTCA. The lyophilized standard contains 50,000 IU. A seven-member panel of the reconstituted WHO HCV standard was constructed by gravimetric dilution with HCV-negative plasma (Basematrix diluent; BBI Diagnostics, Boston, Mass.) and included HCV at the following concentrations: 500, 2,500, 5,000, 10,000, 25,000, 50,000, and 75,000 IU/ml. Twelve replicates of the panel member with 75,000 IU/ml, 20 replicates of the panel members with 500, 2,500, 5,000, and 10,000 IU/ml were randomly tested by OTCA in a blinded manner along with 28 negative controls consisting of normal human plasma in two separate runs.

RESULTS

The percentage of individuals actively infected with HCV, as demonstrated by detection of HCV core antigen and HCV RNA in anti-HCV antibody-positive clinical specimens from the treatment-naïve population evaluated in the present study, is shown in Fig. 1. Of note, 110 of 478 (23%) of the specimens showed no evidence of active infection by any of the three assays, while 368 of 478 (77%) of specimens showed evidence of active infection by one or more of the assays. The prevalence of active infection among the seropositive individuals in our study population was similar to that observed in other studies (1, 18, 34). HCV core antigen was detected by OTCA in 331 of 478 (69%) specimens, and HCV RNA was detected by the quantitative HCV bDNA assay in 348 of 478 (73%) specimens. Testing by the more sensitive qualitative HCV TMA detected HCV RNA in an additional 19 specimens, of which 2 were reactive for HCV core antigen by OTCA and 17 were not reactive for HCV core antigen by OTCA. This yielded an overall rate of HCV RNA detection of 76.8% (367 of 478 specimens). The reported quantities of HCV core antigen in the two specimens that had detectable HCV RNA by HCV TMA but in which the viral load was below the limit of quantification of the HCV bDNA assay were 268 and 3.3 pg/ml, respectively. In addition, a third specimen was reactive for HCV core antigen with a quantity of 1.7 pg/ml, just above the 1.5-pg/ml assay cutoff, but was not reactive for HCV RNA by either the HCV bDNA assay or HCV TMA. These results highlight the excellent specificity of OTCA, since only 1 of 331 (0.3%) anti-HCV-positive specimens that were reactive for HCV core antigen had no evidence of HCV RNA. However, OTCA showed a lower sensitivity, since HCV core antigen was not detected in 37 of 367 (10%) anti-HCV-positive specimens from treatment-naïve individuals who had active HCV infection, as documented by testing for HCV RNA. Therefore, the detection of active infection would have been missed in 37 of
704 and 55,000 IU/ml), of which 5 specimens that had HCV RNA concentrations between 2.8 and 6.8 log10 IU/ml. HCV core antigen was not detected in 20 specimens that had HCV RNA concentrations between 2.8 and 4.8 log10 IU/ml (704 and 55,000 IU/ml), of which 5 specimens that had sufficient volume for retesting were reactive for HCV RNA by the qualitative AMPLICOR HCV (version 2.0) assay. In addition, HCV core antigen was not detected in 17 specimens that were reactive for HCV RNA by HCV TMA but in which the virus was present at levels below the quantification limit of the HCV bDNA assay (and, therefore, whose data are not shown on the histogram). Eleven of these 17 specimens had sufficient volume for retesting by the qualitative AMPLICOR HCV (version 2.0) assay, of which 7 were reactive for HCV RNA, 1 was indeterminate for HCV RNA by two successive tests, and 3 were not reactive for HCV RNA. Of the three specimens that were not reactive, two with sufficient residual volume were found to be negative for HCV RNA upon repeat testing by HCV TMA. Taken together, these results show that HCV core antigen was not reliably detected in specimens from patients with active infection when HCV RNA levels were 4.8 log10 IU/ml or less.

The correlation between the HCV core antigen concentration and the HCV RNA load for 328 specimens in which active infection was detected by both OTCA and the HCV bDNA assay is shown in Fig. 3. For this analysis, 72 of 328 (22%) of the specimens tested had absorbance values above the linear range of OTCA and thus required dilution and retesting. By comparison, only 1 of 328 (0.3%) of the specimens required dilution and retesting by the HCV bDNA assay. Deming regression analysis of these 328 specimens yielded the equation $y = 0.98x - 3.59$ with a correlation coefficient of 0.915, where $y$ is the concentration of HCV core antigen in log10 picograms per milliliter and $x$ is the concentration of HCV RNA in log10 international units per milliliter. This equation indicates that the relationship between the concentrations of HCV core antigen and HCV RNA was consistent throughout the common dynamic ranges of the assays.

The LoD of OTCA was estimated by testing a dilution panel of the WHO HCV standard (Fig. 4). The results showed that OTCA detected core antigen in 5 of 24, 20 of 20, 19 of 20, and 12 of 12 replicates of dilutions with estimated HCV RNA concentrations of 10,000, 20,000, 50,000, and 75,000 IU/ml, respectively. No core antigen was detected in dilutions containing 5,000 IU of HCV RNA per ml or less. These data were
analyzed by linear regression analysis to determine the LoD, or the analyte detection sensitivity, which is defined as the minimum analyte concentration at which the assay could distinguish a reactive specimen from a nonreactive specimen 95% of the time. This definition of LoD has been recommended in the National Committee for Clinical Laboratory Standards protocol for qualitative molecular methods (26). The x coordinate where the calculated curve intersects the 95% detection line indicates that the estimated LoD of OTCA is 27,000 IU/ml (95% CI, 20,000 to 49,000 IU/ml).

DISCUSSION

Proper clinical diagnosis and management of HCV-infected individuals require both serological screening for anti-HCV antibodies and qualitative HCV RNA testing (27). Despite the declining incidence of HCV infections in North America over the past 15 years, the prevalence of HCV infections worldwide continues to pose a significant disease burden; more patients are expected to be diagnosed with the disease in the next two decades because of the long latent period prior to the first clinical signs of liver impairment (18). Hence, diagnostic laboratories will likely face increasingly high demands for accurate diagnostic screening and confirmation of active HCV infection.

As the largest reference laboratory in Canada on the basis of the population served, BCCDC performs approximately 95% of the HCV antibody tests for the 4 million inhabitants of the province of British Columbia. In the past year, BCCDC performed 96,000 anti-HCV antibody tests, of which approximately 4,800 (5%) showed some degree of reactivity and therefore required a second test for confirmation of active HCV infection. Although qualitative HCV RNA testing by an assay with a sensitivity of 50 IU/ml is the laboratory’s method of choice at present, given the large volume of specimens tested annually, we wished to explore simpler technologies that could be used to confirm active HCV infection. As a potential alternative to HCV RNA testing, we evaluated OTCA as a second-line confirmatory test for the diagnosis of active HCV infection. OTCA offers a number of practical advantages: (i) it can be performed in less time than HCV RNA tests; (ii) it does not require specific training in molecular techniques; and (iii) as an EIA, it is anticipated that OTCA might be less expensive than HCV RNA tests. Also, OTCA is theoretically not as susceptible to carryover contamination as HCV RNA tests and can therefore be performed with the same sample used for the initial anti-HCV antibody test. However, a second aliquot would still be required to minimize the risk of HCV RNA carryover if core antigen-negative specimens are to be reflexed for additional qualitative HCV RNA testing.

It is important to evaluate an assay in the context of its intended use, since an assay may provide great utility in one setting but less utility in another. To our knowledge, ours is the first study to evaluate OTCA as a supplemental test in the diagnostic setting once seroconversion has been confirmed. Previous studies have evaluated the utility of OTCA for the screening of blood products, focusing on its potential to shorten the time to the identification of active infection prior to antibody production during the window period (12, 16, 17). Other studies have examined the assay’s utility in the monitoring of therapy, as assessed by its ability to assess the presence or the absence HCV core antigen at a given time point or to examine the kinetics of HCV core antigen decline (22, 32, 36, 37). Many of the previously published studies reported on the performance of an earlier prototype version of OTCA, which purportedly is less sensitive than the present version evaluated here. Although a number of the earlier studies have estimated the sensitivity of the assay, none have defined the LoD by assessing multiple dilutions and multiple replicates of a WHO specimen panel.

Our findings show that OTCA was unable to confirm active HCV infection in a significant proportion of specimens. No core antigen was detected in 30.8% of specimens tested for confirmation of active infection, of which 25.2% were shown to be viremic by HCV RNA testing. Hence, if OTCA were used as a second-line test by using the current cutoff recommended by the manufacturer, at least 30.8% of specimens would have required testing for HCV RNA to confirm active HCV infection. Although HCV core antigen was detected in all samples of HCV viremic patients, the viremic specimens in which core antigen was detected had HCV RNA levels up to 48,600 IU/ml.

Our correlations between the concentrations of HCV core antigen (in picograms per milliliter) and the concentrations of
HCV RNA (in international units per milliliter) are also consistent with those reported in earlier studies. Bouvier-Alias et al. (6) reported correlations of 1 pg of HCV core antigen per ml to 7,688 IU of HCV RNA per ml for genotype 1b and to 6,711 IU of HCV RNA per ml for genotype 3. Similarly, the correlations estimated from the differences between the mean concentrations of HCV core antigen and HCV RNA for each genotype reported by Veillon et al. (36) suggest that 1 pg of HCV core antigen per ml corresponds to HCV RNA concentrations of 8,128 IU/ml for genotype 1, 8,511 IU/ml for genotype 2, and 9,550 IU/ml for genotype 3. The variation among the reported correlations between HCV core antigen and HCV RNA concentrations may be explained in part by the use of different quantitative assays and specimens from different patient populations in the various studies. While our study used the third-generation HCV bDNA assay, Veillon et al. (36) used the second-generation version of this assay and the AMPLICOR HCV MONITOR test (version 2.0; Roche Molecular Diagnostics), and Bouvier-Alias et al. (6) used the third-generation HCV bDNA assay as well as the AMPLICOR HCV MONITOR test (version 2.0) and the SuperQuant assay (National Genetics Institute, Culver City, Calif.). Even though the quantification results were expressed in the same international units, the quantification results reported from the different HCV RNA assays may not be equivalent (25). Also, while our study population consisted exclusively of treatment-naive patients, the studies of Veillon et al. (36) and Bouvier-Alias et al. (6) included patients undergoing therapy. Not only do the mean values for HCV core antigen and HCV RNA levels differ between these patient groups, but the relationship between HCV core antigen and HCV RNA concentrations may also vary between patients (6, 16). For all of these reasons, the reported correlations between the concentration of HCV core antigen and the HCV RNA concentration may vary from study to study.

The relatively poor sensitivity of OTCA compared to those of nucleic acid-based tests accounts in part for its inability to detect core antigen in Acrometrix OTCA in its present and earlier formats. In evaluating a prototype version of OTCA for which the manufacturer set a less sensitive cutoff of 2.0 pg/ml, Bouvier-Alias et al. (6) found that the assay consistently detected core antigen in Acrometrix panel members containing ≥200,000 IU of HCV RNA per ml but in only one of nine replicates of a panel member containing 50,000 IU of HCV RNA per ml. Veillon et al. (36) evaluated the present version of OTCA, for which the manufacturer has set a lower detection limit of 1.5 pg/ml, by testing dilutions of the WHO standard and found that the assay detected core antigen in three of three replicates of each of the 1:4, 1:2, and 1:1 dilutions containing estimated HCV RNA concentrations of ~12,500, ~25,000, and ~50,000 IU/ml, respectively. Our study also tested dilutions of the WHO standard and determined a LoD for OTCA of 27,000 IU/ml (95% CI, 20,000 to 49,000 IU/ml), which represents the viral level at which OTCA detects core antigen in HCV RNA-positive specimens 95% of the time. The LoD should not be confused with the manufacturer’s cutoff, which is not necessarily based on the 95% detection limit. The LoD of 27,000 IU/ml that we determined is consistent with our findings of the ability of OTCA to detect core antigen in clinical specimens. Most viremic specimens (36 of 37 [97%]) in which core antigen was not detected had HCV RNA levels below 27,000 IU/ml, although 1 specimen that was not reactive for core antigen had a viral level of 55,000 IU of HCV RNA/ml.

At its present level of sensitivity, OTCA has limited value as a second-line confirmatory test for the diagnosis of active HCV infection because a significant percentage of viremic individuals have viral loads below its detection limit. OTCA did not detect core antigen in 10% (37 of 367) of viremic samples or in 7.7% (37 of 478) of all samples from the entire population. Given that nucleic acid-based tests are 2 to 4 orders of magnitude more sensitive than OTCA, they are better able to detect viremia. For example, the limit of quantification of the HCV bDNA assay is 615 IU/ml, and the LoD of the quantitative AMPLICOR HCV MONITOR test (version 2.0) is 600 IU/ml (20) and the LoD of the qualitative AMPLICOR HCV (version 2.0) test is 50 to 100 IU/ml (15). AMPLICOR HCV test, version 2.0; Roche Molecular Systems, 2001 [http://www.fda.gov/cdrh/pdf/p000010.html]. Therefore, at present qualitative nucleic acid-based testing remains the standard for the confirmation of active HCV infection in the diagnostic setting.

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