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Comparison of Hepatitis C Virus RNA and Antibody Detection in Dried Blood Spots and Plasma Specimens

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Abstract

Background—Current diagnostic tests for Hepatitis C Virus (HCV) involve phlebotomy and serologic testing for HCV antibodies (anti-HCV) and RNA, which are not always feasible. Dried blood spots (DBS) present a minimally invasive sampling method and are suitable for sample collection, storage and testing.

Objectives—To assess the utility of DBS in HCV detection, we evaluated the sensitivity and specificity of DBS for anti-HCV and HCV RNA detection compared to plasma specimens.

Study design—This cross-sectional validation study was conducted in the context of an existing prospective study of HCV in young injection drug users. Blood samples were collected by venipuncture into serum separator tubes (SST) and via finger stick onto Whatman 903® protein-saver cards. Plasma samples and eluates from the DBS were tested for anti-HCV using either a third generation enzyme-linked or chemiluminescent immunoassay (IA), and HCV RNA using discriminatory HCV transcription-mediated amplification assay (dHCV TMA). DBS results were compared to their corresponding plasma sample results.

Results—148 participants were tested for anti-HCV and 132 participants were tested for HCV RNA. For anti-HCV, the sensitivity of DBS was 70%, specificity was 100%, positive predictive value (PPV) was 100%, negative predictive value (NPV) was 76% and Kappa was 0.69. For HCV

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Ethical Approval
Study protocols and procedures were reviewed and approved by the UCSF Committee for Human Subjects Institutional Review Board.

RNA, the sensitivity of DBS was 90%, specificity was 100%, PPV was 100%, NPV was 94% and Kappa was 0.92.

Conclusions—DBS are sensitive and very specific in detecting anti-HCV and HCV RNA, demonstrate good correlation with plasma results, and have potential to facilitate diagnosis of HCV infection.

1. Background

Hepatitis C virus (HCV) infection is the most common blood-borne infection in the world and a major cause of morbidity globally.[1,2] Approximately three percent of the world's population has been infected with the virus and there are up to 170 million people with chronic HCV infection[3] who are not only at increased risk for developing cirrhosis and hepatocellular carcinoma, but also pose a risk for the continued spread of infection. In the United States (U.S.), 1,229 cases of acute HCV were reported in 2011, and these numbers represent an estimated 16,500 actual acute infections.[4] There are an estimated 4.1 million persons in the U.S. with antibodies to HCV,[5] indicating acute or chronic infection with the virus. About half of incident HCV infections occur in people who inject drugs (PWID), previously referred to as injection drug users, which likely represents a significant underestimate of the true percent of infection attributable to injection drug use (IDU) exposure, due to underreporting and limited surveillance.[6]

Among persons living with HIV, coinfection with HCV predicates worse clinical outcomes, including increased HCV viral load, hepatic fibrosis, more rapid progression to cirrhosis and end-stage liver disease,[7] and reduced response to HCV treatment.[8] High rates of HIV/HCV coinfection are found among PWID, with up to 80% of HIV-positive PWID co-infected with HCV in some areas,[9,10] and reports of increasing HCV incidence in HIV-infected men who have sex with men.[11] Despite this, evidence suggests that people living with HIV are not routinely screened for HCV infection.[12]

In the U.S. it is estimated that up to 75% of persons infected with HCV are unaware of their infection status.[13] Testing to ascertain HCV infection status currently involves testing for both HCV antibodies (anti-HCV) and HCV RNA to correctly diagnose infection, since anti-HCV testing does not distinguish acute, chronic or resolved infection. Acutely infected individuals may be viremic for up to two months before development of antibodies.[14] On average, 25% of persons infected with HCV will spontaneously resolve infection,[15] most within six months after infection.[16] Without testing for HCV RNA, persons who are being tested for HCV infection cannot know their actual infection status. With accurate diagnosis of HCV, acutely infected patients can benefit from early initiation of therapy which significantly increases the likelihood of disease clearance.[17] With new and more effective HCV treatments becoming available and in order to reduce HCV associated morbidity and mortality, diagnosis of HCV infection is now an emerging health priority.[18]

To accurately diagnose HCV infection, patients often undergo several phlebotomy procedures for the multiple tests. These procedures often pose a challenge for PWID, the population most at risk for HCV, who are typically regarded as ‘hard sticks’, and may be a deterrent for testing in general. Among some populations in international settings, blood

draws are not culturally acceptable and as a result, HCV testing is not prioritized. Current testing protocols may also limit HCV diagnosis in situations where venipuncture is not convenient or readily available and in many parts of the world with limited diagnostic technology, making the diagnosis of HCV in resource-constrained settings a challenge. These issues highlight the need for the development of alternative diagnostic testing for both anti-HCV and HCV RNA that requires a minimal amount of blood, is readily available, and is less invasive in the diagnosis of HCV infection. The addition of a new point-of-care test for anti-HCV that has recently become available can help minimize invasive procedures; an important step in making HCV testing more accessible to PWID and other at-risk groups.[19]

Dried blood spots (DBS) present a minimally invasive sampling method that are readily available and facilitate sample collection and storage. DBS involves the collection of capillary blood from a fingerstick onto a protein-saver card, which is then air-dried and stored until ready for processing. DBS have been successfully employed in the diagnosis of HIV and quantification of viral load.[20-22] However, the use of DBS to diagnose HCV using both anti-HCV and HCV RNA has not been validated. The application of DBS for comprehensive HCV testing could increase uptake of HCV counseling and testing as well as aid in early detection and diagnosis of infection among high risk populations. Timely and accurate diagnosis of HCV would identify persons for treatment, and early detection of acute HCV infection may facilitate targeted public health interventions that could help reduce transmission of HCV especially among PWID and other high-risk populations.[23] DBS samples may be stored and transported for testing at a later date, which may also provide enhanced surveillance in resource-limited settings.

2. Objectives

We sought to assess the diagnostic accuracy of using DBS testing for diagnosis of HCV infection, testing for both anti-HCV and HCV RNA. Our specific aims were to determine the sensitivity and specificity of DBS in anti-HCV and HCV RNA detection and the implications for improving HCV diagnosis

3. Study design

3.1. Context

This cross-sectional study was conducted in the context of an existing prospective study of HCV in young adult (<30 years old), active (injected in the past 30 days) injection drug users in San Francisco (The UFO Study) for prospective follow up of risk for and natural history of HCV infection. Methods for recruitment and follow up in the UFO Study have been described in detail.[14,16,24,25] In brief, recruitment for the prospective study was done was by outreach workers and by word of mouth. Cohort eligibility was restricted to persons who were English speaking and who did not plan to travel outside of San Francisco within three months of study enrollment. Consenting HCV-negative participants were enrolled into follow up to examine risks for and incidence of HCV infection. Participants were scheduled for interviewing, HCV testing, and pre-test and post-test counseling every three months. Those who became HCV infected were followed monthly. Socio-demographic information and risk exposures were obtained from participants at each visit using interviewer

administered questionnaires. Participants were tested for both anti-HCV and HCV RNA at all follow-up visits. For this study, DBS and plasma samples were collected concurrently in participants presenting for baseline screening between July 2010 and June 2011.

3.2. DBS Specimen collection and storage

Trained phlebotomists performed a finger stick on each eligible study participant using a lancet device and collected 0.5ml of whole blood onto Whatman 903® protein-saver cards. The blood spots were air-dried for two hours, then packaged and sent to Blood Systems Research Institute (BSRI) in San Francisco, where they were stored at < −70°C. The dried blood spots were sent to Creative Testing Solutions in Arizona for laboratory analysis.

3.3. DBS Specimen elution and processing

DBS processing—A thorough literature review was performed to help determine appropriate DBS size and elution processes to use. The DBS controls were prepared using a 6.35 mm hole punch. The hole punch was cleaned and prepared prior to each use by spraying with 10% bleach solution, wiped down, cleaned with an alcohol prep consisting of 70% Isopropyl Alcohol, and allowed to air dry prior to performing each punch. The Whatman 903® protein-saver cards were kept on dry ice during the punch process. Each control was prepared separately using the hole punch and a process to limit any potential for contamination. The punch required for testing was placed in a cryovial and brought to room temperature while the unused cards were returned to −80°C.

Preparation of controls—Controls were prepared using 5 NAT and HCV enzyme-linked immunosorbent assay (ELISA) positive whole blood samples to be used as the known positive controls and 5 NAT and HCV ELISA nonreactive samples to be used as known negative controls. The discriminatory HCV transcription-mediated amplification assay (dHCV TMA) sensitivity panel was made by serial dilution of one of the known positive controls. Three anti-HCV ELISA weakly positive controls and one serial dilution of a strong positive control were used for the anti-HCV 3.0 sensitivity panel.

Whole blood controls were brought to room temperature and then gently inverted 5 to 10 times to mix blood thoroughly. After blood was completely mixed, the cap was removed with a blood bloc, and then 50 μL of whole blood was applied to each spot on the Protein saver card for that control. The 5 blood spots on the card were allowed to air dry overnight without flap over the spots in a clean dry place that was protected from direct sunlight. The cards were then folded closed and placed into a sealable gas impermeable zip lock bag containing desiccant packs. No more than one card was stored per bag and they were stored at −80°C until required for testing.

Elution preparation—The controls were then used to evaluate different elution methods for both dHCV and HCV Version 3.0 ELISA testing to determine the optimal elution method for sample testing. [26] Additional testing was performed on the eluates including an overnight incubation of eluate and immediate freezing of eluate to see if a modified storage method could be performed.

A PBS Elution was prepared by adding 125μL of PBS (Dulbecco's Phosphate Buffered Saline x1) to one 6.35 mm DBS. The DBS vials were shaken for 10 minutes then incubated overnight at 2-8°C. After incubation, the supernant was used for testing both dHCV and HCV ELISA tests. A dHCV Elution was prepared by adding 500 μL distilled water plus 400 μL Target Capture Reagent to DBS and then was incubated at 60°C for 60 minutes. After incubation, the supernant was used to perform subsequent extraction and detection of dHCV. An HCV ELISA Elution was prepared by adding 200 μL specimen diluent (SD). The DBS vials were mixed and incubated overnight at 2-8°C. After incubation, the eluate was used to perform HCV ELISA testing.

dHCV TMA and HCV Version 3.0 ELISA testing—Eluates from the DBS were tested for HCV RNA using a standard dHCV TMA (Norvatis®). Clinical sensitivity for the assay has been demonstrated for specimens with HIV-1 or HCV viral RNA concentrations ≥ 100 copies/mL. dHCV testing consisted of 25 positive control and 25 negative control tests using the PBS and dHCV elution methods. The best correlation for the dHCV TMA testing was observed with the PBS Eluate tested the next day. Evaluation of the controls resulted in 1 false positive when the PBS Eluate was immediately frozen and 1 false positive with the dHCV Eluate.

Eluates from the DBS were tested for anti-HCV using ELISA v3.0 (Ortho®). The specificity of ELISA in anti-HCV detection among a low prevalence population of blood donors is 99.95%. The sensitivity in detection of anti-HCV among patients with acute infection is 75.3%, and 88.1% among patients with chronic infection. HCV ELISA testing consisted of 25 positive control and 25 negative control tests using the PBS and HCV ELISA elution methods. HCV ELISA testing showed the best correlation with initial screening reactivity in SD Eluate when it was tested the next day. Evaluation of the HCV ELISA 3.0 using 25 positive controls and 25 negative controls resulted in 3 false positive results among the negative controls when the entire PBS eluate was used. Among the weakly reactive samples in the sensitivity panels were 3 false negatives. Based on the control testing it was decided that the same DBS preparation for study samples would be used, as contamination was not observed. Additionally, it was determined that the PBS elution tested the next day would be used for dHCV and the SD elution tested the next day would be used for HCV ELISA.

The DBS test results were compared to the corresponding plasma sample test results for anti-HCV and HCV RNA from the cohort study. Plasma specimens were tested for anti-HCV using either a third generation enzyme-linked or chemiluminescent immunoassay (IA) and for HCV RNA using the Procleix® TMA Assay.[14] In cases where anti-HCV plasma specimens were unavailable on the DBS test date (n=13), positive plasma specimens immediately prior or negative plasma specimens immediately after DBS testing were compared to DBS results.

3.4. Statistical Analysis

Sensitivity, specificity, positive predictive value, negative predictive value and kappa of DBS compared to plasma specimens for anti-HCV and HCV RNA were analyzed using SAS 9.3®.

4. Results

DBS and plasma specimens from 148 participants were tested for anti-HCV and specimens from 132 participants underwent HCV RNA testing. The DBS results were compared to corresponding plasma sample results from the same patient, using the plasma result as the gold standard.

Of the 148 specimens that underwent HCV EIA testing, 77 (52.0%) were anti-HCV positive based on testing of plasma specimens and 54 (36.5%) were positive based on testing of DBS samples. 71 (48.0%) were anti-HCV negative based on testing of plasma specimens and 94 (63.5%) were negative based on DBS testing.

For anti-HCV testing, the sensitivity of DBS testing is 70.1% (95% CI 58.5-79.8), specificity is 100% (95% CI 93.6-100), positive predictive value (PPV) is 100% (95% CI 91.7-100), negative predictive value (NPV) is 75.5% (95% CI 65.4-83.6) and kappa is 0.69 (95% CI 0.0.58-0.81) (Table 1).

Of the 132 specimens that underwent dHCV TMA testing, 48 (36.4%) were HCV RNA positive based on testing of plasma specimens and 43 (32.6%) were positive based on testing of DBS samples. 84 (63.6%) were HCV RNA negative based on testing of plasma specimens and 89 (67.4%) were negative based on DBS testing.

For HCV RNA testing, the sensitivity of DBS testing is 89.6% (95% CI 77.8-95.5), specificity is 100% (95% CI 95.6-100), PPV is 100% (95% CI 91.8-100), NPV is 94.4% (95% CI 87.5-97.6), false positive rate (α) is 0%, false negative rate (β) is 5.6% and kappa is 0.92 (95% CI 0.84-0.99). (Table 2)

We calculated the mean signal-to-cutoff ratio (S/Co) of the dHCV TMA results to determine if they differed by concordance between DBS and plasma results (Table 3). The mean was lower for the five discordant specimens (19.19; 95% CI 6.79-31.59) compared to the forty-three concordant results (24.26; 95% CI 23.70-24.81), and the p-value for the t-test is <0.01. S/Co data for the ELISA 3.0 test are not entered in our database, limiting our ability to calculate mean values from anti-HCV testing.

5. Discussion

HCV infection is a public health problem of global significance. Standard HCV diagnostic tests involve testing plasma specimens for anti-HCV and HCV RNA, which is not always feasible, or acceptable to some patients. Consequently, many cases of acute and chronic HCV infection go undiagnosed. The aim of this study was to assess the diagnostic accuracy of a non-invasive testing strategy using DBS to detect HCV. To accomplish this we estimated the sensitivity and specificity of HCV test results from blood sample droplets collected on filter paper, compared to current testing for anti-HCV and HCV RNA using venous blood samples among young IDU.

There are limited data on comparative assay performance using DBS for detection of HCV. In this study, different elution and storage methods were compared to optimize HCV RNA

and Ab detection. dHCV TMA testing showed the best correlation with reactivity in PBS Eluate tested the next day, and HCV ELISA testing showed the best correlation with initial screening reactivity in SD Eluate tested the next day.

For anti-HCV testing, only 54 of the 77 HCV EIA positive plasma results were positive by DBS. Using the plasma results as the gold standard, DBS correctly identified 70% of the positive tests as positive (true positives) and falsely classified 30% of the positive tests as negative (false negatives). All of the HCV EIA negative plasma results were negative by DBS, reflecting a specificity of 100%. The kappa of 0.69 demonstrates 'good' strength of agreement between the DBS result and the plasma result. For HCV RNA testing, 43 of the 48 dHCV TMA positive plasma results were positive by DBS. Using the plasma results as the gold standard, DBS correctly identified approximately 90% of the positive tests as positive and falsely classified 10% of the positive tests as negative. All of the dHCV TMA negative plasma results were negative by DBS, reflecting a specificity of 100%. The kappa of 0.92 demonstrates 'very good' strength of agreement between the DBS result and the plasma result.

A comparison of mean S/Co of dHCV TMA results demonstrated that the mean is lower for discordant results compared to concordant results, but still well above the level predictive of a true positive test ≥95% of the time for different screening tests, regardless of the population being tested or the prevalence of HCV in the population. [27]

We tested specimens from PWID, a population with high HCV prevalence, which could have contributed to the high positive predictive values obtained. In addition, our results included 13 specimens where anti-HCV plasma samples were unavailable on the DBS test date, and DBS results were instead compared to positive plasma specimens immediately prior or negative plasma specimens immediately after DBS testing. In spite of these limitations, the results of this study demonstrate the feasibility of using DBS for anti-HCV and HCV RNA detection. HCV EIA and dHCV testing of DBS provide sensitive and very specific results when compared to plasma specimens, and these findings are in accordance with a recent study on detection of HCV antigens and antibodies in DBS. [28] Based on minimal invasiveness, ease of sample collection and storage, DBS have the potential to facilitate the detection of HCV antibodies and RNA. This may lead to early diagnosis, reduced risk behaviors and better treatment effects, with an overall improvement in health outcomes. There is currently a highly accurate point of care test for anti-HCV and these results show that it may be possible for HCV RNA testing to be conducted in tandem with this test, which would greatly facilitate the timing of HCV diagnosis. This validation study was limited by the small number of samples tested. Future studies may better assess the accuracy of DBS for anti-HCV and HCV RNA by comparing a larger number of DBS to plasma specimens.

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review. AA participated in cohort study supervision and manuscript review. AB participated in cohort study supervision and manuscript review. KP conceptualized the study, participated in study design and manuscript review. All authors approved the final content of the manuscript.

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Table 1

Sensitivity, specificity, positive and negative predictive values of DBS compared to plasma specimen for anti-HCV

Anti-HCV	Plasma Positive	Plasma Negative	Total
DBS Positive	54	0	54
DBS Negative	23	71	94
Total	77	71	148

Sensitivity = 70.1% (95% CI 58.5-79.8)

Specificity = 100% (95% CI 93.6-100)

Positive Predictive Value = 100% (95% CI 91.7-100)

Negative Predictive Value = 75.5% (95% CI 65.4-83.6)

Kappa = 0.69 (95% CI 0.58-0.81)

Table 2

Sensitivity, specificity, positive and negative predictive values of DBS compared to plasma specimen for HCV RNA

HCV RNA	Plasma Positive	Plasma Negative	Total
DBS Positive	43	0	43
DBS Negative	5	84	89
Total	48	84	132

Sensitivity = 89.6% (95% CI 77.8-95.5)

Specificity = 100% (95% CI 95.6-100)

Positive Predictive Value = 100% (95% CI 91.8-100)

Negative Predictive Value = 94.4% (95% CI 87.5-97.6)

Kappa = 0.92 (95% CI 0.84-0.99)

Table 3

Mean signal-to-cutoff ratio (S/Co) of dHCV TMA results by concordance between DBS and plasma results

Concordance	n	Mean (SD[a])	Test value[b]	p-value
Discordant	5	19.19 (9.99)	−3.14	0.0029
Concordant	43	24.26 (1.80)		

[a] Standard deviation.

[b] Independent t-test value.