Disparate detection outcomes for anti-HCV IgG and HCV RNA in dried blood spots

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ABSTRACT

Dried blood spots (DBS) expedite the collection, storage and shipping of blood samples, thereby facilitating large-scale serologic studies. We evaluated the sensitivity of anti-HCV IgG testing and HCV-RNA quantitation using freshly prepared and stored DBS derived from HCV-infected patients.

Protocols for elution were optimized using DBS prepared from plasma of 52 HCV-infected persons and 51 uninfected persons (control DBS), then applied to DBS from 33 chronic hepatitis C patients that had been stored at -20°C for 5 years (stored DBS). Control and stored DBS, and their corresponding plasma, were processed for anti-HCV IgG testing using the VITROS chemiluminescence assay (CIA) and the HCV 3.0 enzyme immunoassay (EIA) (Ortho-Clinical Diagnostics), and for HCV RNA quantitation by quantitative (q) RT-PCR. HCV genotyping was conducted by nucleotide sequencing.

The sensitivity of CIA and EIA in control DBS was 92% and 90% respectively, compared to 100% and 97%, respectively, in stored DBS. The sensitivity of HCV RNA detection was 88% in control DBS, compared to 36% in stored DBS. Specificity was 100% for all the assays in both control and stored DBS. Genotypes 1, 2 and 3 were detected in 16 (62%), 6 (23.1%), and 4 (15.3%) samples, respectively. Sequences generated from DBS and their corresponding plasma samples were identical.

Whereas the sensitivity of anti-HCV IgG detection in stored DBS was equivalent to that in recently prepared DBS, the sensitivity of HCV RNA detection was markedly lower in stored DBS compared to recently prepared DBS. Stored DBS may be reliably used for anti-HCV detection but for HCV-RNA-based testing freshly prepared DBS is preferable to stored DBS.

KEYWORDS: hepatitis C antibodies, blood specimen collection, real-time polymerase chain reaction, molecular typing Genotype

1. Introduction

Hepatitis C virus (HCV) currently infects approximately 150 million persons worldwide (World Health Organization (WHO), 2014) and 3.2 million persons in the United States (Holmberg et al., 2013). HCV infection can cause acute hepatitis and chronic infection associated with long-term liver disease. Persons with chronic HCV infection run the risk of developing cirrhosis, liver cancer, or both. The diagnosis for HCV infection is based on serological testing for anti-HCV IgG and HCV RNA. The preferred clinical specimen for detecting these markers is serum or plasma, pre-analytic processing of which can be challenging for field studies especially in remote or resource-poor settings.

Dried blood spots (DBS) were originally used to collect blood to screen for neonatal congenital and metabolic disorders (Guthrie et al., 1963; Therrell and Adams, 2007), and have been used for detecting infection by pathogens such as cytomegalovirus (Atkinson et al., 2009), herpes simplex virus (Hogrefe et al., 2002) and human immunodeficiency virus (Ross et al., 2013), and identification of immunoglobulin subtypes (Anderson et al., 2014). Obtaining DBS is minimally invasive as blood may be drawn by skin pricking rather than venipuncture (CLSI NBS01-A6, 2013). Subsequent centrifugation and refrigeration are not required (Lew et al., 1998). In addition, DBS are amenable to storage owing to the flatness of the filter paper cards (Alvarez-Muñoz et al., 2005), further facilitating shipping, and minimize required storage volume (http://www.cdc.gov/labstandards/pdf/nsqap/Bloodspot_Transportation_Guidelines.pdf). These advantages expedite large-scale serologic studies.

The potential use of DBS as an alternate sample to serum or plasma for anti-HCV IgG testing and HCV RNA detection has been explored in several studies, with variable sensitivity and specificity outcomes (Tuaillon et al., 2010; Santos et al., 2012; Brandao et al., 2013; Shepherd et al., 2013; Dokubo et al., 2014). Anti-HCV IgG testing in DBS has been shown to be more affordable and practical for screening high-risk populations. However, there is a paucity of data on the utility of DBS for detection of anti-HCV IgG or HCV RNA in both fresh and stored samples using well defined positive and negative control DBS samples. The outcomes are described of anti-HCV and HCV RNA testing in DBS freshly prepared from plasma samples of uninfected and HCV-infected patients and in DBS from HCV-infected persons that had been stored long-term.

2. Materials and Methods

2.1. DBS processing

Identical procedures for DBS were employed for comparing elution protocols using a diluent comprising 150 mM NaCl, 50mM sodium phosphate, pH 7.4, with 0.05% Tween-20 (PBS/T), and the diluent of the VITROS anti-HCV assay (Ortho Clinical Diagnostics, Rochester, NY). DBS was eluted from two 12-mm spots fully excised by a hole-puncher, resulting in a total of ten 3-mm disks, transferred directly into an Eppendorf microtube containing 500 μ l of either PBS/T or VITROS sample diluent. Each microtube containing the disks and the buffer was briefly vortexed, incubated at 55°C for 10 minutes and then shaken at 4°C overnight. To maximize elution from DBS, the elution buffer and each soaked disk were transferred to a 3-ml vacutainer syringe (BD, Franklin Lakes, NJ) connected to an uncapped 2-ml cryovial (Fisher Scientific, Pittsburgh, PA) as previously described and illustrated (Mercader et al., 2006). The eluate was recovered by applying pressure with the syringe plunger to the buffer and the disks. Eluates were collected from the cryovials once the disks were white, indicating complete elution of blood proteins, and then were stored at -20°C until used. Each DBS contains approximately 75 μ l of blood, of which 41 μ l would be plasma, assuming a normal hematocrit. Therefore, elution of two DBS in 500 μ l of buffer results in 1:6 dilution of plasma.

To avoid carry-over contamination, disposable tips were used to transfer the eluate and move the disks to the 3-ml vacutainer syringe; individual hole-punchers for each spot were used. After each set of experiments the hole-punchers were decontaminated with 70% ethanol and RNase zap (Life Technologies, Grand Island, NY).

2.2. Control DBS Preparation

DBS positive and negative control samples were freshly prepared by mixing O+ red blood cells (RBCs) purchased from Tennessee Blood Services (Memphis, TN) with HCV-RNA-positive plasma samples and HCV-RNA-negative sera. Before mixing, the units of RBCs were washed 3 times with saline to remove plasma, buffy coat, and anticoagulant as described previously (Mei et al., 2001). The washed RBCs were packed by low speed centrifugation to obtain a hematocrit of least 95% RBCs. The corresponding amounts of well characterized plasma samples from donors (CSL Behring, King of Prussia, PA) were added to aliquots of packed RBCs to produce a hematocrit of 45% for each sample. The samples were then re-suspended and spotted onto Grade 903 filter paper (GE Healthcare, Piscataway, NJ) at 100 μ L blood per spot. The DBS sample set was prepared from 10 anti-HCV-positive/HCV-RNA-negative, 42

anti-HCV-positive/HCV-RNA-positive, 38 anti-HCV-negative/HCV-RNA-positive, and 13 anti-HCV-negative and HCV-RNA-negative plasma donors (total 103).

2.3. Stored DBS and corresponding plasma samples

DBS and corresponding plasma samples from 33 adult patients with chronic hepatitis C at the Liver Clinic of the University of California, San Francisco, were collected from August 2007 to July 2008. The study was approved by the ethical boards of University of California San Francisco (UCSF) (#00000229; Federal Wide Assurance (FWA)#00000068) and the US Centers for Disease Control and Prevention (CDC). Whole blood was collected by phlebotomy from which plasma was fractionated, and capillary blood from finger-prick was spotted on Grade 903 filter paper. Study specimens were stored with desiccant (GE Healthcare) inside Bitran[®] bags (Fisher Scientific, Pittsburgh, PA) at –20°C until use 5 years later.

2.4. Anti-HCV IgG testing

Two immunoassays, the VITROS anti-HCV IgG chemiluminescence assay (CIA) and the HCV 3.0 enzyme immunoassay (EIA), both from Ortho Clinical Diagnostics (Rochester, NY), were used to detect anti-HCV in control and stored DBS samples with their corresponding plasma. DBS eluates were mixed 1:10 (v/v) with diluent, achieving a 1:60 final dilution of plasma; this additional dilution was applied in CIA and EIA. Reproducibility of CIA and EIA for DBS were evaluated by performing an inter-assay variability test. Signal-to-cutoff ratio (SCR) was used to measure anti-HCV IgG reactivity in the stored and control DBS and their corresponding plasma samples. A reproducibility panel of DBS eluates was assembled to include a high, medium, and low anti-HCV-IgG reactive sample based on the SCRs from each assay. Samples were tested in triplicate by the same operator for three days using the same kit lot. In the reproducibility test, three control DBS samples with known anti-HCV SCRs were tested in triplicate on three different days. High, medium, and low anti-HCV-positive samples were selected based on their SCRs: respectively, 29.8, 10.4 and 3.26 in CIA, and 6.4, 4.2 and 1.5 in EIA.

2.5. HCV RNA extraction and detection by quantitative PCR

Fifty microliters of total nucleic acid (TNA) were extracted from 200 µl of the DBS eluate using MagNA Pure LC 2.0 (Roche Diagnostics, Indianapolis, IN), and then processed for two-step, qRT-PCR (Mixon-Hayden et al., 2014) using the Superscript III Platinum 1 Step qRT-PCR kit, with 10 µl of TNA in a total volume of 25 µl. Primers and TaqMan probe are presented in table 1. In order to quantitate HCV RNA in DBS, 10-fold serial dilutions of the Acrometrix HCV RNA Panel (Life Technologies, Grand Island, NY) were

spiked into the RBC from an HCV-negative donor, and then used to create DBS for the standard curve (Figure 1; efficiency 2.443, slope -2.577, and error 0.0168). To determine the stability of HCV RNA in control DBS at different storage temperatures, an HCV-RNA-positive plasma sample, containing 6.79 log₁₀ IU/mL of HCV RNA was serially diluted using HCV-RNA-negative human serum (Sera Care Life Sciences, Milford, MA) to spike the RBC preparations, and then spotted onto Grade 903 filter paper cards. HCV RNA titer was measured in eluates from DBS stored at -20°C, 4°C, 22°C, and 37°C for 60 days with desiccant inside Bitran® bags.

2.6. Nucleotide sequencing studies

All HCV-RNA-positive samples were re-amplified for sequencing by nested PCR targeting the 5'UTR with the Qiagen One-Step RT-PCR kit (Qiagen, Valencia, CA) using 24 µl TNA extract in 50-µl reaction volumes with primers F15 and R03 (Table 1). Second-round PCR was performed using Quanta SybrGreen Master Mix (Quanta BioSciences, Gaithersburg, MD) using 2 µl of the primary product in 20-µl final volumes with primers F13 and R04 (Table 1). Amplification was performed using the Light Cycler® 480 software (Version 1.5.0.SP3, Roche Diagnostics, Indianapolis, IN). PCR conditions included incubation at 94°C for 150 s, followed by 40 cycles of 94°C for 20 s, 45°C for 25 s and 72°C for 45 s. Nested PCR with 30 cycles was performed on 1 µl of the first-round product using the same conditions. Second-round PCR products were sequenced using nested primers. Sequencing reactions were conducted using the BigDye v3.1 chemistry sequencing kit (Life Technologies, Grand Island, NY), and products were sequenced using a 3130xl Genetic Analyzer (Life Technologies, Grand Island, NY). Sequencing PCR involved 25 cycles, each cycle consisting of 96°C for 10 s, 50°C for 5 s and 60°C for 2 min. Preliminary sequence analysis was conducted using SeqMan and MEGALIGN programs from the Lasergene DNA and Protein analysis software (version 9.0, DNASTAR , Madison, WI). The 5'UTR sequences were aligned using Geneious Pro. Version 5.5.8 (Biomatters, New Zealand) and also with MUSCLE as implemented in Mega5 (Tamura et al., 2004). Phylogenetic trees were constructed using the maximum-likelihood algorithm (Tamura et al., 2004) implemented in MEGA 5.0. Bootstrap values were calculated using 1000 replicates. HCV genotypes were assigned by comparing each sequence with 15 known GenBank reference sequences: AB442219, AF165045, GQ418224, HM043176, JF34876, JQ273008, JQ745652, KC197238, KC844050, KC844052, KF279472, U01214, U45476, Y11604, and Y12083.

2.7. Sensitivity and Specificity

Sensitivity and specificity were determined by comparing the results of anti-HCV and HCV RNA in DBS eluates to the results of their corresponding plasma samples. Sensitivity was defined as the number of positive reference DBS samples that were also positive in the corresponding plasma samples (true-positive) divided by that number plus the number of negative reference DBS samples that were positive in the corresponding plasma samples (false-negative).

Specificity was defined as the number of DBS negative reference DBS samples that were also negative in the corresponding plasma samples (true-negative) divided by that number plus number of positive reference DBS samples that were negative in the corresponding plasma samples (false-positive).

3. RESULTS

3.1. Elution process and anti-HCV IgG testing

The anti-HCV reactivity of DBS serial dilutions in PBS/T and the VITROS specimen diluent buffers were not statistically different (data not shown). Accordingly, further evaluations were conducted using PBS/T as an elution buffer.

For control DBS samples, the sensitivity of CIA was 92% (48 DBS samples anti-HCV reactive /52 corresponding plasma samples anti-HCV reactive) and for EIA was 90% (47 DBS samples anti-HCV reactive/ 52 corresponding plasma samples anti-HCV reactive). The corresponding plasma specimens of the DBS eluates that tested negative yielded low SCRs, ranging from 1.25 to 5.45.

For stored DBS, the sensitivity for CIA was 100% (all 33 samples tested anti-HCV reactive in both DBS and their corresponding plasma samples), and for EIA was 97% (32 DBS samples detected/33 plasma samples detected).

Specificity for both CIA and EIA, evaluated only in control DBS, was 100% (all 51 DBS and their corresponding plasma samples tested negative).

3.2. HCV RNA detection in control and longtime stored DBS

The potential loss of HCV RNA titer in DBS versus plasma over time was evaluated in control and stored specimens. Twenty-five stored DBS were chosen to match control DBS based on comparable levels of anti-HCV-IgG expressed in SCR, as well as HCV RNA concentration in the corresponding plasma samples. The average HCV RNA concentration in paired plasma of both stored and control samples was 6.3 log₁₀ IU/ml (range: 2.8-7.2 log₁₀ IU/ml). HCV RNA was detected in 9 (33%) of the stored DBS and 22 (88%) of the control DBS samples. The average HCV RNA concentration loss of 4.4 log₁₀ IU/mL (range: 0-4.6 log₁₀ IU/ml) was observed between stored DBS samples and their corresponding plasma samples, whereas that for control DBS samples and their paired plasma samples was 2.5 log₁₀ IU/mL (range: 0-3 log₁₀ IU/ml).

3.3. HCV RNA sequencing in control and longtime stored DBS

Twenty-six DBS eluates and their corresponding plasma were sequenced to determine the HCV genotype. The genotype distribution of these 26 pairs was: 1a (n=6), 1c (n=10), 2 (n=6) and 3 (n=4). Sequences generated from DBS and their corresponding plasma samples were identical.

3.4. Stability of HCV RNA in DBS at different storage temperatures

HCV RNA concentration loss between fresh DBS and plasma after 60 days storage was observed (Figure 2). The neat concentration for the study plasma sample was 7.6 log₁₀ IU/mL stored at -20°C, 7.3 log₁₀ IU/mL at 4°C, 5.3 log₁₀ IU/mL at 22°C, and 3.1 log₁₀ IU/mL at 37°C. The HCV RNA concentration detected in stored DBS was 5.5 log₁₀ IU/mL at -20°C, 4.7 log₁₀ IU/mL at 4°C, 3.7 log₁₀ IU/mL at 22°C, and 2.7 log₁₀ IU/mL at 37°C.

3.5. Inter-Assay Evaluation

The intra-assay means and standard deviation of freshly engineered DBS using the CIA and EIA are presented in Table 2. The overall percent covariance (%CV), was 2.61 (95% CI, 0-4.29) for CIA and 2.61 (95% CI, 0-5.81) for EIA.

4. Discussion

DBS eluates are useful test samples for detection of serological markers of infection with various pathogens, especially in resource-limited areas. The handling and collection of DBS are less labor-intensive and require fewer resources. The Guthrie card was first used for phenylketonuria screening of newborns in the 1960s (Guthrie et al., 1963). Since then, other uses have been explored. DBS has been used to screen for HIV infection, allowing for large-scale screening and monitoring. The use of DBS allows for collections of specimens to be shipped without refrigeration to a better-resourced laboratory for confirmatory testing and additional analysis.

In this study, markers of HCV infection were tested in two sets of DBS: those from freshly-prepared (control) DBS samples and those from chronic hepatitis C patients stored for 5 years (stored DBS samples). Anti-HCV was detectable in eluates from control and stored DBS, with eluates from stored DBS showing similar detection efficiencies as their corresponding plasma specimens. However, four eluates from freshly prepared DBS tested false negative by CIA and five for EIA; one eluate from stored DBS tested negative by EIA. The corresponding plasma specimens of these DBS eluates had low SCRs, ranging from 1.25 to 5.45. Thus, DBS eluates tested negative perhaps due to 1:6 dilution resulting from the elution process. The lower sensitivity of testing anti-HCV in DBS versus plasma is consistent with the data reported by Ross et al. (2012). In previous studies, the anti-HCV detection sensitivities from DBS samples have ranged between 70-100% (Tuailon et al., 2010; Santos et al., 2012; Brandao et al., 2013; Shepherd et al., 2013; Dokubo et al., 2014) with specificities from 94% to 100%. In our study, the sensitivity for control and stored DBS ranged between 92%-100%, and specificity was 100%.

The sensitivity of HCV RNA detection in control DBS was 88% (95% CI, 69%-97%). For stored DBS the sensitivity of HCV RNA detection was only 36% (95% CI, 18%-57%), these findings suggest RNA degradation over time. Between plasma and corresponding freshly-prepared DBS, a 3-log₁₀ IU/mL decline was observed. Since these are all eluates that were directly recovered from the blood spot, the low sensitivity may originate from compounds in the blood that inhibit PCR (Al-Soud et al., 2001). Regardless of the reduced sensitivity of HCV RNA detection in DBS eluates, there was complete identity between sequences derived from DBS and plasma.

Contrasting with the findings of Tuailon et al. (2010), this study reported HCV RNA degradation when DBS were exposed to ambient room temperature for over 4 weeks. In this study, HCV RNA detectability in control DBS samples remained relatively stable for at least 60 days or when stored at -20°C, 4°C and 22°C, but declined by ~3 log₁₀ IU/mL at 37°C.

Use of DBS for serology and nucleic acid testing has several limitations: the stability issues in case of improper collection, drying, storage and transportation; and the laborious steps during which disks need to be manually excised and eluates diluted, leading to lower sensitivity. To avoid cross-contamination during the re-filing of the residual DBS, the samples must be properly labeled and handled one at a time; weighing paper and humidity indicator must be replaced. Despite these limitations, use of DBS would offer a convenient and cost-effective alternative to phlebotomy, obviating the need to separate serum or plasma and to transport along a cold chain.

In conclusion, this study shows that anti-HCV IgG was detected in DBS eluates with similar efficiency as in plasma and it can be detected in DBS eluates after they have been stored for several years; whereas the sensitivity of anti-HCV IgG detection in stored DBS was equivalent to that in recently prepared DBS, the sensitivity of HCV RNA detection was markedly lower in stored DBS compared to freshly prepared DBS. Thus, stored DBS may be reliably used for anti-HCV detection, but for HCV-RNA-based testing freshly prepared DBS is preferable to DBS that had been stored long-term.

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Table 1. Oligonucleotide sequences of HCV RNA detection and sequencing primers.

Purpose	Primer/Probe	Sequence (5'-3')
Detection	HCV146U	GTC TGC GGA ACC GGT GAG
	HCV253R	CGA CCC AAC RCT ACT CGG CTA G
	HCV165P*	AC ACC GGA ATT GCC AGG ACG ACC
Sequencing	F15	CTGTGAGGAACTACTGTCT
	R03	GTGCACGGTCTACGAGACCT
	F13	GAAAGCGTCTAGCCATGGCGT
	R04	CCCTATCAGGCAGTACCACAA

*TaqMan Assay (Tag) Probe

Table 2. Inter-assay reproducibility of Ortho HCV 3.0 EIA (A) and VITROS anti-HCV CIA (B) in high-positive-, medium-positive-, and low-positive- DBS specimens, one specimen per category, tested in triplicate over 3 days.

(A) Reproducibility of VITROS anti-HCV CIA

Sample*	%CV		
	Day 1	Day 2	Day 3
High-positive	2.18	4.29	4.16
Medium-positive	3.48	0	2.62
Low-positive	2.97	3.82	0
Negative	0	2.48	4.88

Overall %CV (range): 2.61 (0-4.29)

(B) Reproducibility of Ortho HCV 3.0 EIA

Sample**	%CV		
	Day 1	Day 2	Day 3
High-positive	0	0	0
Medium-positive	5.81	3.64	5.66
Low-positive	0	3.51	2.51
Negative	35.36	28.57	32.64

Overall % CV (range): 2.35 (0-5.81)

*CIA: High-positive (SCR $\geq$ 20), medium-positive (8<SCR<20), and low-positive (1<SCR <8)

**EIA: High-positive (SCR $\geq$ 5), medium-positive (3.8<SCR<5), low-positive (0.9<SCR<3.8)

Figure 1. DBS Standard curve. A neat sample representing the standard DBS with RNA concentration of $6 \log_{10}$ IU/mL was used to prepare 10-fold dilutions, indicated by points along the standard curve.

Figure 2. HCV RNA stability in plasma vs. DBS eluates. Both specimens were stored at -20°C, 4°C, 22°C and 37°C for 60 days with desiccant and bitran bag. Plasma specimen contained 7.6 log₁₀ IU/mL of HCV RNA, and DBS eluate contained 5.5 log₁₀ IU/mL.