



Detection of hepatitis C virus RNA in dried blood spots

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ARTICLE INFO

Article history:

Received 11 January 2012

Received in revised form 6 February 2012

Accepted 8 February 2012

Keywords:

HCV
HCV RNA
Dried blood spot
RT-PCR
Intravenous drug user

ABSTRACT

Background: An estimated 130–170 million people worldwide are chronically infected with HCV.¹ In Europe the highest prevalence of HCV infections is in the IDU population.² As traditional HCV screening relies on the detection of HCV antibody or HCV RNA in blood, screening in high-risk groups such as IDU is difficult due to poor venous access caused by damaged veins.

Objectives: In this study DBS was evaluated as an alternative sample type to blood for the detection of HCV RNA.

Study design: The endpoint detection limit, inter-assay and intra-assay variability of the method were determined. The DBS method was compared to our routine frontline assay using a panel of paired DBS and blood samples. The effect of different storage temperatures and length of storage time on the stability of HCV RNA in DBS was also assessed.

Results: The endpoint detection limit of the method based on results from mock DBS was 250 IU/ml. The method was shown to be precise and robust. The sensitivity and specificity of the method was found to be 100% and 95.8%, respectively. No significant variation in the stability of HCV RNA in DBS over a 1 year period at a range of different temperatures was observed.

Conclusions: A sensitive and stable method was developed for the detection of HCV RNA in DBS. Screening high-risk populations using DBS as a sample type may improve uptake of HCV testing by increasing opportunity for patients to be tested and consequently increasing access to treatment.

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1. Background

Worldwide it is estimated that 130–170 million people are chronically infected with HCV, with 3–4 million new infections per year.¹ In Europe the highest prevalence of HCV infections is in the IDU population. However most carriers are unaware of their infection status,² for example recent estimates suggest that in Scotland 58% of current/former IDU's infected with HCV remain undiagnosed.³

As traditional HCV screening relies on the detection of HCV antibody or HCV RNA in blood, screening in high-risk groups such as IDU is difficult. This high-risk population often have chaotic lives and are unlikely to attend clinics where trained phlebotomists can take blood for screening, and even if patients do attend they are

likely to have poor venous access due to damaged veins, therefore venipuncture may not be possible.

In order to increase screening of populations such as this, alternative and less invasive sampling types to serum/plasma for HCV testing have been evaluated. Oral fluid has been used to detect a number of viral infections including HBV, HCV and HIV. It is easy to self administer and collect, and the sample can be sent via post to the laboratory.^{4–16} However this sample type is not suitable for diagnostic testing^{17–19} as studies have shown that HCV RNA in saliva is independent of the viral load,²⁰ and patients with low serum HCV loads were less likely to have detectable HCV in their saliva.²¹

Another non-invasive sample type is the collection of DBS from finger tips. This sampling method is less invasive than venipuncture causing little discomfort to the patient, therefore encouraging individuals to come forward for screening. DBS collection does not require trained phlebotomists, and DBS can also be transported and stored easily without the need for cold chain transport.

Several papers have been published showing DBS as a suitable alternative sample type to serum/plasma for the detection of HCV antibody.^{10,22–28} However there have been few studies published evaluating the use of DBS for the detection of HCV RNA.^{29–33}

Abbreviations: DBS, dried blood spot; HCV, hepatitis C virus; HBV, hepatitis B virus; HIV, human immunodeficiency virus; RNA, ribonucleic acid; IDU, intravenous drug user; RT-PCR, reverse transcriptase polymerase chain reaction; Ct, cycle threshold.

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Furthermore, some have suggested a loss in sensitivity of HCV RNA if DBS are stored at room temperature for 4 weeks or more.³¹

2. Objectives

In this study we evaluated DBS as an alternative sample type to blood for the detection of HCV RNA.

Several aspects were examined. Firstly, the endpoint detection limit was assessed. Following this the inter-assay and intra-assay variability of the method was determined. The DBS method was then compared to our routine frontline assay using a panel of known HCV positive antibody samples. Finally we assessed the effect of different storage temperatures and length of storage time on the stability of HCV RNA in DBS to address the issues raised by Tuailon et al.³¹

3. Study design

3.1. Samples

3.1.1. Preparation of DBS for endpoint detection limit

Mock DBS were made by diluting NIBSC 2nd HCV RNA international standard [WHO, HPA UK] in negative blood to give dilutions of 1000 IU/ml, 500 IU/ml, 250 IU/ml and 150 IU/ml. 50 µl of each dilution was then spotted onto Protein Saver™ 903® Cards [www.whatman.com], filling the spot and ensuring that the blood fully saturated through the filter paper. The cards were then dried at room temperature for at least 1 h. The mock DBS were stored at 4 °C until use and tested using the DBS method described below.

3.1.2. The inter-assay and intra-assay variability

The inter-assay and intra-assay variability of the method was also assessed. The inter-assay variability (reproducibility) was assessed by repeat testing of individual 1000 IU/ml spot controls over 20 PCR runs. The intra-assay variability (repeatability) was assessed by testing a 1000 IU/ml spot control in 20 wells on one PCR run.

3.1.3. Prospective testing of paired DBS and blood samples

The DBS method was compared to our frontline assay using 80 known HCV antibody positive paired DBS and blood samples taken from patients at the same visit. All patients were attending an HCV specialist clinic and PCR status was mixed.

3.1.4. Stability of DBS over time under different storage conditions

To assess the effects of different storage conditions on the stability of HCV RNA in DBS, a set of DBS were made from a blood sample of a known HCV RNA positive individual. The DBS had an initial average ct of 26.04 and were assessed for stability at different temperatures (room temperature (~21 °C), 4 °C, -20 °C, -80 °C). Two DBS specimens were extracted and tested in duplicate (giving 4 ct (cycle threshold) values) on day one and then at weekly intervals until week 4, then after at 6 weeks, 2 months, then at monthly intervals until 6 months, then at 9 months and 12 months.

3.1.5. Laboratory methods

3.1.5.1. DBS extraction protocol. The DBS specimens were eluted using a protocol for RNA extraction from DBS verified by van Deursen et al.³² provided by bioMérieux (Lyon, France). Briefly, one full DBS (estimated 50 µl whole blood) was cut out and placed into a tube containing 2 ml of NucliSENS lysis buffer (bioMérieux). At this stage an RNA internal control (IC) grown in-house (equine arteritis virus) was added. The tubes were then incubated on a roller bank for 30 min at room temperature, then centrifuged at 1500 × g for 15 s and the spots removed. 1.7 ml of the eluate was used for

Table 1
Inter-test variability of the method.

	Mean CT	SD	Max	Min	CoV
1000 IU/ml	33.01	0.83	35.50	30.51	0.0251

SD: standard deviation. Max and min are ±3 standard deviation. CoV: co-efficient of variation.

extraction on the bioMérieux easyMag according to the manufacturer instructions and nucleic acid was eluted in a 25 µl volume.

3.1.5.2. Extraction of blood samples. Blood samples were extracted using the Abbott M2000 extraction platform, 600 µl of serum/plasma was extracted and nucleic acid eluted in a 60 µl volume.

3.1.5.3. Real time PCR for HCV detection. HCV and IC were amplified using an in-house HCV/IC duplex real time RT-PCR assay. The HCV primer and probe sequences and the internal control (EAV) assay has been published previously.^{34,35} The PCR assay in conjunction with extraction of HCV RNA on the Abbott m2000 extraction platform has a detection limit of approximately 50 IU/ml in serum/plasma samples. This assay detects all HCV genotypes.

For blood testing 600 µl of blood is extracted and the RNA is eluted into a volume of 60 µl (10 fold concentration), whereas for DBS testing an estimated 50 µl of blood is eluted into a volume of 25 µl (2 fold concentration). Therefore, there is 5 fold less starting volume of blood with DBS than blood, and so the endpoint detection of DBS should be 250 IU/ml.

Real Time TaqMan RT-PCR was performed using reagents from Quanta BioSciences (Gaithersbury, USA). A 2-step RT-PCR was carried out, the qScript™ cDNA synthesis kit (95047-100) was used for the reverse transcriptase step and then for amplification the PerfeCta® qPCR SuperMix (95063-500) was used. cDNA was synthesised using 11.25 µl of RNA extract in a 15 µl reaction and the following thermal profile: 22 °C for 5 min, 42 °C for 30 min, 85 °C for 5 min, and 4 °C hold on an ABI 9700 (Applied Biosystems, Warrington, UK). PCR was then performed on 6 µl of cDNA in a 15 µl reaction using the following thermal profile: 50 °C for 2 min, 2 min at 95 °C for reverse transcriptase inactivation and DNA polymerase activation followed by 40 amplification cycles of 8 s at 95 °C and 34 s at 60 °C (annealing-extension step) on an ABI 7500 (Applied Biosystems, Warrington, UK). This PCR kit is specifically designed for use with multiplex assays to reduce competition. Kit volumes and thermal profiles were adapted in-house.

4. Results

4.1. Endpoint detection limit

100% of 1000 IU (18/18), 100% of 500 IU/ml (18/18), 100% of 250 IU/ml (10/10) and 70% of 150 IU/ml (7/10) dried NIBS spots were detected, suggesting that the endpoint detection limit for detection of HCV RNA from DBS using the method described is between 150 and 250 IU/ml.

4.2. Inter-assay and intra-assay variability

We assessed inter-assay variability (Table 1), and intra-assay variability (Table 2). The results suggest that there is little inter-assay variability as the Ct of the 1000 IU/ml control was similar over 20 different runs with low standard deviation values (standard deviation of 0.83) and low co-efficient of variation (C_V) values. The results also suggest that the intra-assay variability of the assay is also good as little variation was observed when the positive control was repeatedly tested (standard deviation of 0.73) and low (C_V)

Table 2
Intra-test variability of the method.

	Mean CT	SD	Max	Min	CoV
1000 IU/ml	32.42	0.73	34.61	30.24	0.0225

SD: standard deviation. Max and min are ± 3 standard deviation. CoV: co-efficient of variation.

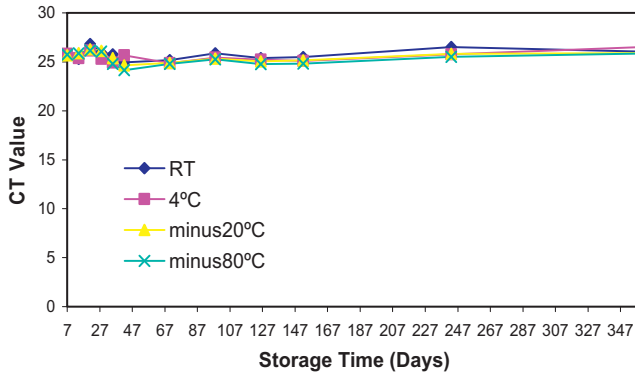


Fig. 1. Stability of RNA in DBS over time under different storage conditions.

Table 3
Stability of RNA in DBS over time under different storage conditions.

Temperature	Mean CT	SD	CoV
RT	25.72	0.561	0.020
4°C	25.51	0.490	0.016
–20°C	25.51	0.514	0.021
–80°C	25.31	0.641	0.026

SD: standard deviation; CoV: co-efficient of variation; RT: room temperature ($\sim 21^\circ\text{C}$).

values. Overall these results suggest that the duplex assay is precise and robust.

4.3. Evaluation of paired DBS and blood samples

In order to directly compare the performance of HCV RNA detection from DBS to serum/plasma samples 80 known antibody positive paired DBS and blood specimens were tested by both methods. Of these 57 found to be RNA positive in serum/plasma, all 57 were also found to be positive in DBS, giving a sensitivity of 100%. Of the 23 samples found to be RNA negative in serum/plasma, 22 were found to be negative in DBS therefore 1 false positive was detected in DBS (ct > 35), giving a specificity of 95.8%.

4.4. Stability of DBS under different storage conditions

We examined the stability of DBS at room temperature ($\sim 21^\circ\text{C}$), 4°C , -20°C and -80°C over a period of 12 months. We observed no significant variation in the stability of HCV RNA in DBS over this period irrespective of different storage temperatures (Fig. 1). There was little variation between ct values irrespective of storage temperature or length of storage as shown by a low standard deviation and co-efficient of variation values (Table 3).

5. Discussion

In this study, we evaluated a method for the extraction and detection of HCV RNA from DBS.

The endpoint detection limit of the method based on results from mock DBS was found to be 250 IU/ml. The DBS method was

also shown to be precise and robust by assessment of the inter-assay and intra-assay variability. The endpoint detection limit of this method is lower than other methods reported in the literature. For example De Crignis et al.²⁹ extracted RNA from DBS using the manual kit QIAamp DNA mini kit and SYBR green PCR and obtained an endpoint sensitivity of 500 IU/ml. Brown et al.³⁰ used the bioMérieux easyMag and in-house PCR to obtain a detection limit of 1000 IU/ml, and similar results were found by Tuailon et al.³¹ used the COBAS extraction and PCR to obtain a detection limit of approximately 1000 IU/ml.

Although not as sensitive as the frontline assay, the sensitivity of the method will enable detection of HCV in most cases. Retrospective analysis of new HCV diagnoses undertaken between 1st January and 30th June 2010 at the West of Scotland Specialist Virology Centre showed that approximately 1% (7/540) of these had RNA detected below 250 IU/ml.³⁸

One way to overcome this is to request a repeat sample, either blood or DBS, to confirm any antibody positive and RNA negative DBS, therefore allowing detection of any weak samples that are weak RNA positive.

Of concern was the one false positive sample in the paired DBS and blood panel. The ct value of the sample was over 35, and the sample was from a sample previously and known to be HCV RNA negative and on repeat testing the DBS was negative. Subsequent samples were also found to be RNA negative. One explanation for this false positive could be cross-contamination.

To monitor for contamination one blank spot for every 7 DBS samples is processed and extracted. In addition a no template control (water) is added to the PCR plate. Over the course of the study, no cross contamination was observed. Another explanation could be PCR degradation resulting in spurious positives, or DBS quality.³⁶ As mentioned previously, all DBS positive results are repeated with a follow-up sample to confirm positive results.

Overall our method was found to be a sensitive alternative to blood testing which would prove to be useful in screening hard to reach populations such as IDU's which may in turn increase testing. For example Hickman²⁴ reported an average increase of 14.5% in HCV testing in the intervention sites where DBS were offered compared to their matched controls using venipuncture alone.

As described in the introduction some recent publications have suggested DBS degrade rapidly at room temperature resulting in reduced sensitivity of the sample type which leads to false negative results.³¹ To address this we assessed the effect of different storage conditions and times of the stability of DBS. We found no evidence of HCV RNA degradation in DBS stored under different conditions over time. Similarly Cassol and Solomon^{36,37} found no loss in HCV RNA stability over time in DBS. However these results contradict those found in a recent publication by Tuailon et al.³¹ where they found that prolonged exposure (4 weeks) at room temperature impaired RNA recovery, and also a decrease in assay specificity. Abe et al. reported a 10-fold reduction in viral yield from dried serum spots after 4 weeks at room temperature using a method of manual extraction and conventional PCR.

In summary, we have developed and evaluated a sensitive and stable method for the detection of HCV RNA in DBS. This study shows DBS to be a sensitive alternative to blood testing. Screening high-risk populations using DBS as a sample type may improve uptake of HCV testing by increasing opportunity for patients to be tested and consequently increasing access to treatment.

Funding

Scottish hepatitis C Action Plan.

Competing interest

None.

Ethical approval

West Glasgow Ethics Committee GN09V1207.

Acknowledgments

We appreciate the support of the following colleagues:

Co-investigators: Colin Geddes, Ray Fox, Peter Mills, Adrian Stanley, Ann Muir Greater Glasgow & Clyde HB. Paul McIntyre, John Dillon Tayside HB. The Research Nurses from Western Infirmity Clinical Trials Unit.

Co-workers: Gillian Mulholland, Elizabeth Spence, Margaret Neilson from GGCHB and Jan Tait from Tayside HB.

Laboratory technicians: Anthony Bimpson, Linda MacDonald, Douglas Condie and Malgorzata Walkowicz, West of Scotland Specialist Virology Centre, Glasgow.

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