

## Application of Dried Blood Spot Testing For Hepatitis C Virus RNA Amplification

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### ABSTRACT

**Background/Aim :** Hepatitis C Virus (HCV) infection represent a major public health problem because of the ability of HCV to cause a chronic carrier state. Even though chronic carriers remain largely asymptomatic, a large number of these individuals subsequently develop cirrhosis and primary hepatocellular carcinomas. Dried Blood Spot (DBS) samples are a simple and inexpensive sampling method, especially useful for blood collection in resource poor settings with limited access to diagnostic facilities. The main advantage of DBS samples over routine blood samples is that only a small quantity of blood is required, easy to obtain, stable and can be transported to a reference laboratory at minimal cost. **The Aim of the work** was to evaluate the feasibility of DBS samples as an alternative sample type to serum for the detection of HCV RNA. Results obtained from DBS samples were compared with results of serum using the same technique. **Methods:** This study was carried out on 50 anti-HCV-positive serum samples, from patients whom attending Arar Central Hospital and Prince Hospital, Arar, kingdom of Saudi Arabia., during November 2011 to February 2012. **Results:** HCV RNA was detected in 49/50 (98%) of the DBS samples, with Sensitivity 98% and Specificity 100 %, in comparisons to serum samples. Also there was no statistical significant difference in hepatitis C viral load between the two different samples among the patients. We demonstrated that there is no statistical significant different between the two samples when viral load is both less than and also more than 100000 IU. **Concluded** that the use of DBS for extraction and amplification of HCV RNA was reliable, specific, sensitive, cheap and appropriate method to monitor the HCV infected patients.

### INTRODUCTION

Hepatitis c virus (HCV) represents a major health problem with approximately 3% of the world population that is more than 170 million people infected. While only 20–30% of individuals exposed to HCV recover spontaneously, the remaining 70–80% develops chronic HCV infection (CHC)<sup>[1]</sup>. Moreover, 3–11% of those people will develop liver cirrhosis (LC) within 20 years<sup>[2]</sup>, with associated risks of liver failure and hepatocellular carcinoma (HCC)<sup>[3]</sup> which are the leading indications of liver transplantation in industrialized countries. The socioeconomic impact of HCV infection is therefore tremendous and the burden of the disease is expected to increase around the world as the disease progresses in patients who contracted HCV years ago<sup>[4]</sup>.

Routine screening for HCV infection relies on detecting antibodies against HCV (anti-HCV) using highly sensitive second- or third-generation enzyme immunoassay. However, the diagnosis of acute or chronic infection also requires the detection of HCV RNA by polymerase chain reaction (PCR)<sup>[5]</sup>, likewise, when recent HCV infection is suspected or the patient is immunocompromised, then the sample

should be referred for PCR. Samples with a low screening signal-to-cutoff ratio may also need confirmation with these more specific recombinant immunoblot assays<sup>[6, 7]</sup>.

Some population groups, such as sex workers, the homeless, prisoners, or other institutionalized individuals, have a higher prevalence of HCV infection than the general population<sup>[8,9]</sup>. However, HCV testing in these groups is limited by the poor acceptability or feasibility of veinipuncture. Collecting capillary blood spots on filter paper requires less staff training, is less invasive, involves smaller blood volumes, and is ideal for high-risk patients with damaged veins, such as intravenous drug users<sup>[10]</sup>. In addition, this technique can reduce the cost of HCV testing by simplifying sample collection, processing, storage, and shipment<sup>[11]</sup>.

The World Health Organization (WHO) found dried blood spots a suitable alternative to serum for testing in places where resources are limited. The Centre for Disease control has also developed guidelines for collecting and transporting samples<sup>[12]</sup>.

**The Aim of the work:** was to evaluate the feasibility of DBS samples as an alternative sample type to serum for the detection of HCV RNA. Results obtained from DBS samples were

compared with results of serum using the same technique.

## MATERIALS & METHODS

*Study approval:* This study was approved by the Ethical Committee of the institution. Legal guardians of all subjects gave their written informed consent before participation in this study.

This cross-sectional study included 50 anti-HCV-positive serum samples, from patients whom attending Arar Central Hospital and Prince Hospital, Arar, kingdom of Saudi Arabia., for HCV infection diagnosis and follow up, during December 2011 to May 2012. Ten anti-HCV- negative serum samples were selected as negative controls. Investigations were performed in virology and immunology unit of Arar Central Hospital.

### The patients were subjected to:

1. History taking including (age, sex, source of infection e.g: surgical procedures, dental procedures, blood transfusion, IV drug abusing).
2. Laboratory investigations in the form of: (CBC, Liver function tests including liver enzymes, albumin, total and direct bilirubin, Prothrombin time and concentration, and Kidney function tests).

### Sampling:

Two to three ml anticubital venous blood sample was collected from each subject and placed immediately into sterile vacutainer tube, allowed to clot naturally; the sera were separated and stored at -20°C for further processing.

Fifty µL of capillary blood was taken by finger prick, using single-use disposable lancets and spotted on a Protein Saver™ 903® Card (Whatman) by Pastier pipette to completely fill 12-mm preprinted circular paper disks. It was air dried at room temperature and then placed in plastic locked bag and stored at -20°C for further processing.<sup>[13]</sup>

### Serological assay for anti-HCV:

Detection of antibodies to hepatitis C virus (HCV) were measured in serum using a commercially available ELISA kit (Murex anti-HCV (version 4.0) ref7 F51-01/-02) (Murex Biotech S.A. (Pty) Ltd). Diluted sample was incubated in microwells coated with highly purified antigens which contain sequences from the core, NS3, NS4 and NS5 regions of HCV. During the course of the first incubation any anti-HCV antibodies in the sample will bind to the immobilized antigens. Following washing to remove unbound material, the captured anti-

HCV antibodies were incubated with peroxidase conjugated monoclonal anti-human IgG. During the course of the second incubation the conjugate will bind to antibody immobilized in the first step. After removal of excess conjugate, bound enzyme was detected by the addition of a solution containing 3,3',5,5'-tetramethylbenzidine (TMB) and hydrogen peroxide. A purple colour will develop in the wells which contained anti-HCV positive samples. The enzyme reaction is terminated with sulphuric acid to give an orange colour which is read photometrically. The amount of Conjugate bound, and hence colour, in the wells, was directly related to the concentration of antibody in the sample.

### Extraction of viral RNA from DBS and serum:

Viral Elution from DBS was performed using two 6-mm spots cut from the 12-mm preprinted circle by a puncher. The pieces were suspended in 1.5-mL Eppendorff microtube with 400 µL of buffer prepared extemporaneously (phosphate-buffered saline, 0.05% Tween 20, and 10% bovine serum albumin) and incubated at 4°C for approximately 2 hours under continuous agitation. After centrifugation (20 seconds at 13,000g), the supernatant was collected<sup>[13]</sup>.

Viral RNA was extracted from supernatant of DBS and serum, using the automated QIAamp Viral RNA mini Kit (Qiagen, GmbH) according to manufacture instructions for automatic extraction in QIAcube extractor (Qiagen, GmbH). The sample is first lysed under highly denaturing conditions to inactivate RNases and to ensure isolation of intact viral RNA. Buffering conditions are then adjusted to provide optimum binding of the RNA to the QIAamp membrane, and the sample is loaded onto the QIAamp Mini spin column. The RNA binds to the membrane, and contaminants are efficiently washed away in two steps using two different wash buffers. High-quality RNA is eluted in a special RNase-free buffer. The purified RNA is free of protein, nucleases, and other contaminants and inhibitors. The special QIAamp membrane guarantees extremely high recovery of pure, intact RNA in just twenty minutes without the use of phenol/chloroform extraction or alcohol precipitation. Viral RNA is adsorbed onto the QIAamp silica membrane during two brief centrifugation steps. Salt and pH conditions in the lysate ensure that protein and other contaminants, which can inhibit downstream enzymatic reactions, are not retained on the QIAamp membrane. Viral RNA, bound to the QIAamp membrane, is washed free

of contaminants during two short centrifugation. The use of two different wash buffers, AW1 and AW2, significantly improves the purity of the eluted RNA. Optimized wash conditions ensure complete removal of any residual contaminants without affecting RNA binding. An internal control (Hep. C Virus RG IC) was added to each sample before extraction according to the manufacturer's instructions. This allows the user both to control the RNA isolation procedure and to check for possible PCR inhibition.

**Amplification of HCV RNA by PCR**

Hepatitis C Virus RNA was quantified using the artus® HCV RG RT-PCR Kit), (Qiagen, GmbH) and the real-time PCR Rotor-Gene® Q Instruments (Qiagen, GmbH). Samples were processed in accordance with the manufacturer's instructions, amplified product is detected via fluorescent dyes. These are usually linked to oligonucleotide probes that bind specifically to the amplified product. Monitoring the fluorescence intensities during the PCR run (i.e., in real-time) allows the detection and quantitation of the accumulating product without having to re-open the reaction tubes after the PCR run. Results were displayed on a computer connecting by Rotor-Gene Q software.

**Statistical analysis:**

Statistical analysis was undertaken using SPSS computer software (SPSS Version 16 for Microsoft Windows). Quantitative data are expressed in terms of mean, standard deviation and qualitative data were expressed in number and percent, appropriate statistical tests were used (f-test, and correlation co-efficient "r" test). ROC curve analysis to determine the

diagnostic power of each test. Results were considered to be statistically significant at  $p < 0.05$

**RESULTS**

The results of this study are presented in tables 1-3 and figure 1-3.

This study included 50 anti-HCV-positive serum samples obtained from HCV infection patient. They were 40 male (80%) and 10 female (20%), their mean age was  $38.5 \pm 12.5$  years and ranged from 39 to 52 years.

Hepatitis C Viruses RNA values obtained from DBS were compared with values from serum samples for 50 HCV-infected patients. HCV RNA was detected in 49/50 (98%) of the DBS samples. With Sensitivity 98% and Specificity 100%, the area under ROC curve was 0.988 which also indicates high sensitivity & Specificity (the more the area under the curve the better the test) (Table 1, Fig1).

There were significant positive correlations between viral load among serum samples and those of DBS samples  $r = 0.9$   $p = 0.000$  ( Fig 2).

There was no statistical significant difference in hepatitis C viral load between the two different samples among the patients (DBS, serum)  $p = 0.19$  (Table 2).

As regarded to differences between samples in different levels of viral load, we demonstrated that there is no statistical significant difference between the two samples when viral load is both less than and also more than 100000 IU,  $n = 7$ ,  $t = 0.98$ ,  $P = 0.34$  and  $n = 42$ ,  $f = 0.33$ ,  $P = 0.74$  respectively (table 3, Fig3)

**Table (1): Sensitivity, specificity, PPV, NPV of DBS viral load:**

		serum viral load		Total
		+ve	-ve	
DBS	+ve	49	0	49
	-ve	1	10	11
Total		50	10	60

- Sensitivity = 98%  
- PPV = 100%

- Specificity = 100%  
- NPV = 90.9%

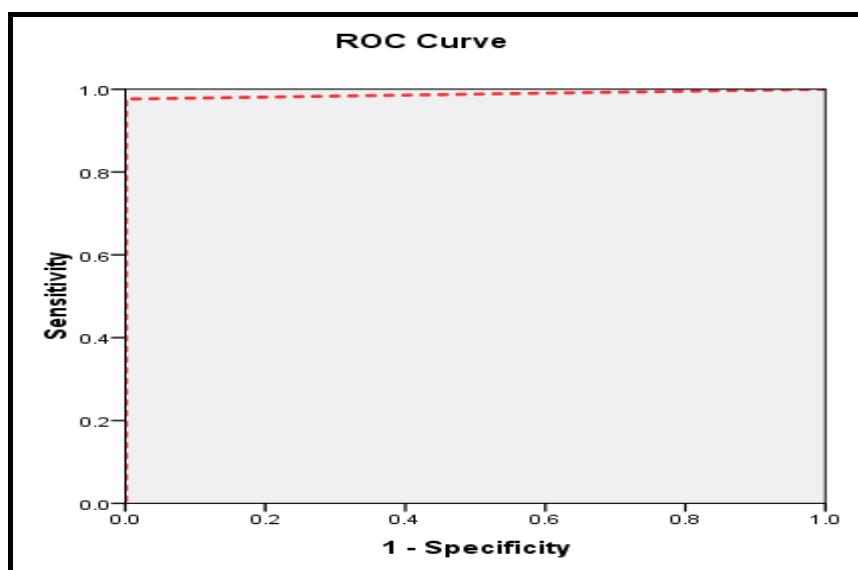


Fig (1): ROC curve for DBS

Area under the Curve

Test Result Variable(s)	Area
DBS viral load	0.988

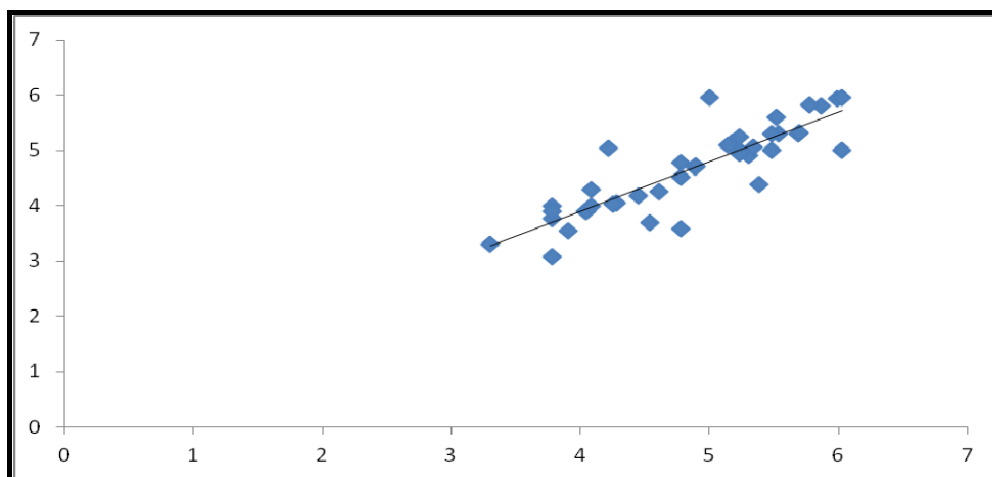


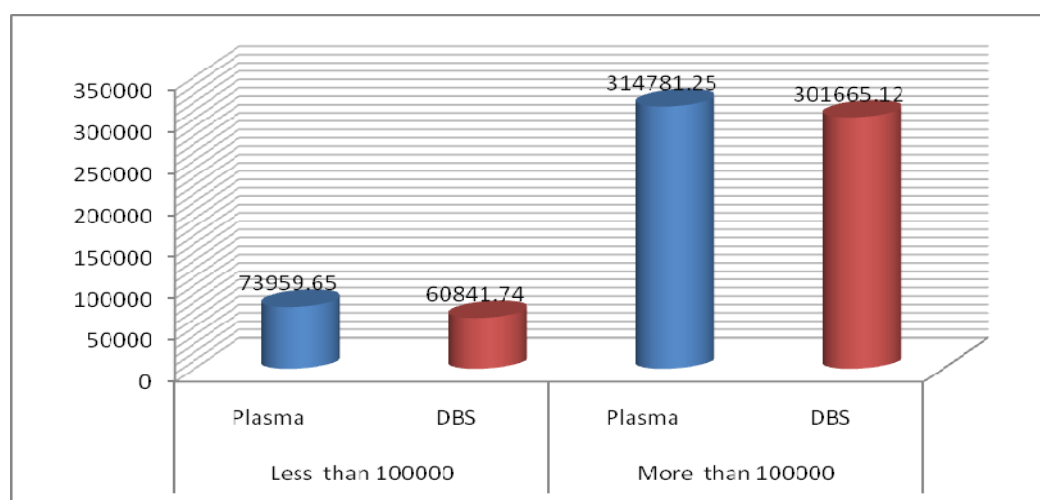
Fig (2): Correlation between DBS viral load, and serum viral load

Table (2): Comparison of the quantitative viral load between groups

	N	Mean	Std. Deviation	t	P
serum	49	4.9	0.74	1.3	0.19
DBS	49	4.7	0.78		

**Table (3): Comparison between two methods in different levels of viral load**

		N	Mean	Std. Deviation	t	P
Less than 100000	Serum	7	73959.65	29984.32	0.98	0.34
	DBS	7	60841.74	18484.42		
More than 100000	Serum	42	314781.25	175495.58	0.33	0.74
	DBS	42	301665.12	190708.47		



**Fig (3): comparison between two methods in different levels of viral load**

### DISCUSSION

Hepatitis C (HCV) is global in distribution. Although the acute presentation of HCV infection is generally mild, often clinically asymptomatic, with only 10 to 25% of patients developing jaundice, For this reason, most patients are not aware of their HCV infection. greater than 50% of infected individuals go on to develop chronic hepatitis with serious and possibly life threatening sequelae such as cirrhosis and hepatocellular carcinoma.<sup>[12,14]</sup>

Collecting capillary blood spots on filter paper requires less staff training , is less invasive , involves smaller blood volume and is ideal for high risk patients with damage veins such as intravenous drug users.<sup>[13]</sup> It is simple sampling method which requires minimal training and the risk of injuries is eliminated as needle and syringe were not used. Since DBS samples do not need special equipment for collection and transportation ,costs are further reduced. This proves to be extremely advantageous for countries with limited health budgets.<sup>[15]</sup>

Dried blood spots have also been shown to be suitable for serology and nucleic acid detection from viral infections including HBV and HIV.<sup>[16,17,18]</sup> Additionally, multiplex real-

time RT-PCR has been used to diagnose hepatitis C/HIV co-infection from DBS samples.<sup>[19]</sup>

The Aim of the work was to evaluate the feasibility of DBS samples as an alternative sample type to serum for the detection of HCV RNA. Results obtained from DBS samples were compared from those from serum using the same technique

In this study we examined the value of DBS in quantifying of HCV. HCV RNA detection was assessed on Whatman 903 cards, which are affordable and readily available worldwide.

The detection of HCV RNA is traditionally carried out by PCR amplification of RNA isolation from serum/plasma<sup>[20]</sup>. since all presently available methods required serum or plasma as a starting material ,blood must be obtained by veinipuncture. In this study we successfully detect and amplify the HCV RNA by using PCR methodology directly on DBS This methods has actually bypass the veinipuncture and plasma/serum separation.

*Hickman et al.*<sup>[21]</sup> reported an average increase of 14.5% in hepatitis C testing in the intervention sites where DBS were offered compared to their matched controls using veinipuncture alone. Other interventions have

reported increases in testing for hepatitis C in the region of 4-6 fold over venepuncture *Craine et al.*<sup>[22]</sup> and *Abou-Saleh et al.*<sup>[23]</sup>

In this study, DBS were compared with values from serum samples for 50 HCV-infected patients and the sensitivity and specificity were 98% and 100 % respectively and this agrees with *Bennett et al.*<sup>[24]</sup> who reported that the sensitivity and specificity of mock DBS was found to be 100% and 95.8%, respectively.

In this study one case was detected by serum sample but not detected by DBS and this can be explained that by very low viral titer measured in this serum sample (339 IU/ml). This result comes true with that reported by *Tuailon et al.*<sup>[13]</sup> who was detecting and quantifying HCV RNA using DBS and found that HCV RNA values obtained from DBS were compared with values from paired serum samples for 62 HCV-infected patients. HCV RNA was detected in 60/62 (97%) of the DBS samples, and in these two DBS with no HCV RNA detected the viral load was (178 and 331 IU/mL) in the matching serum samples, otherwise, there were five DBS samples were positive for HCV RNA but were below the lower limit of detection (15 IU/mL).

In this study there are significant positive correlations between viral load among serum samples and those of DBS samples  $r=0.9$   $p=0.000$  and there is no statistical significant difference in hepatitis C viral load between the two different samples among the patients (DBS, serum)  $p=0.19$  this agrees with *Brunns et al.*<sup>[25]</sup> who reported that, Hepatitis C real-time RT-PCR testing of DBS has been carried out on laboratory prepared „mock“ DBS at the West of Scotland Specialist Virology Centre (WoSSVC). That is, DBS specimens prepared in the laboratory using veinipuncture samples submitted for hepatitis C testing. A pilot study of 50 RNA positive DBS, made from HCV RNA positive blood specimens, gave 100% correlation. also there is a high correlation between hepatitis C virus RNA titre in plasma and small volume capillary blood samples.

There is no statistical significant difference between the two samples when viral load is both less than and also more than 100000 IU,  $n_1=7$ ,  $t=0.98$ ,  $P=0.34$  and  $n_2=42$ ,  $f=0.33$ ,  $P=0.74$  respectively and it is similar to that obtained by *Tuailon et al.*<sup>[13]</sup> who observed a very good concordance between the HCV RNA detected in DBS and serum. The overall sensitivity when using DBS was lower than that for serum in patients with HCV RNA 1,000 IU/mL, but this might have been improved by extracting RNA

from larger DBS samples. also, values below 1,000 HCV RNA IU/mL are very uncommon in untreated patients, and therefore this level of sensitivity may suit pretreatment molecular HCV diagnosis.

In this study blood spot was air dried at room temperature and then placed in plastic locked bag and stored at  $-20^{\circ}\text{C}$  for further processing

*Abe and Konomi*<sup>[26]</sup> observed a 10-fold reduction in HCV RNA titers in dried serum stored at room temperature for 4 weeks. also *Tuailon et al.*<sup>[13]</sup> reported that: for optimal HCV RNA recovery from Whatman 903 paper, DBS should be frozen at  $-20^{\circ}\text{C}$  within 48 hours of collection and drying. Prolonged exposure at room temperature impairs the HCV RNA recovery, whereas recovery was preserved for frozen DBS. These findings are in contrast to the publication by *Solomone et al.*<sup>[27]</sup> who reported no degradation of hepatitis C RNA was found in a set of replicate DBS from 16 hepatitis C RNA-positive patients stored at room temperature and assayed at intervals of 2 to 4 weeks over an 11-month period also *Bennet et al.*<sup>[24]</sup> reported no significant variation in the stability of HCV RNA in DBS over a 1 year period at a range of different temperatures was observed.

## CONCLUSION

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.

Use of DBS for amplification of HCV RNA was reliable, specific, sensitive, cheap and appropriate method to monitor the HCV infected patients. It developed a less invasive, easy and affordable sampling method for those patients. It presented a simple dried viable alternative for routine freezing method for transportation of clinical plasma and serum samples.

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## تطبيق نقطة الدم المجفف فى تضخيم الحمض النووي الريبي لفيروس الكبد سى

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قسم الميكروبيولوجيا والمناعه- قسم الباطنة العامة – قسم الصحة العامة وطب المجتمع- كلية طب بنها

يمثل التهاب الكبدى الوبائى (سي) مشكلة صحية كبيرة مع ما يقرب من ٣٪ من سكان العالم وهذا يمثل أكثر من ١٧٠ مليون شخص مصاب. فى حين أن ٢٠-٣٠٪ فقط من الأشخاص الذين تعرضوا لفيروس (سي) يتعافوا تلقائيا فان ما تبقى من ٧٠-٨٠٪ يتطور المرض ليصل للصورة المزمنة. وان حوالى ٣-١١٪ من هؤلاء الناس يتطور المرض عندهم مسببا لتليف الكبد والمخاطر المرتبطة به من فشل الكبد وسرطانه.

وهدفنا هذه الدراسة الى تقييم استخدام عينات نقطة الدم المجفف كنموذج بديل للسيرم للكشف عن الحمض النووي الريبي سى ومقارنة تلك النتائج بعينات السيرم وذلك باستخدام نفس الطريقة.

وقد شملت الدراسة ٥٠ مريضا بمرضى التهاب الكبدى الوبائى سى بمستشفى عرعر المركزى ومشفى الامير بدمينه عرعر، المملكة العربية السعودية، وكانوا ايجابيون للجسام المضادة للفيروس (سي) وذلك خلال شهر نوفمبر ٢٠١١ إلى ابريل ٢٠١٢. وقد تم اختيار عشرة عينات سلبية للجسام المضادة للفيروس (سي) كضوابط سلبية.

كما تم اخذ ٢-٣ مل دم من الوريد من نفس المريض وفصل السيرم منه بعد تجلته وتخزينه فى -٢٠ درجة مئوية، كذلك وخز اصبع المريض والحصول على ٥٠ ميكرون من الدم رصدت على بطاقة بروتين التوقف (Whatman) ٩٠٣<sup>TM</sup> بواسطة ماصة، تم تعريضها للهواء حتى تجف، ثم وضعها فى كيس من البلاستيك مقفل وتخزينها فى -٢٠ درجة مئوية.

تم استخدام عينات السيرم للكشف عن الاجسام المضادة لفيروس سى عن طريق اختبار الاليزا ثم الاستخلاص الالى للحمض النووي الريبي الفيروسي سى ومضاعفته فى كل من عينه السيرم وعينه نقطه الدم المجفف.

وقد اوضحت النتائج ان ٩٥% (٥٠/٤٩) من عينات نقطة الدم الجافه كانت ايجابيه مقارنة بعينات السيرم بالنسبه لوجود الحمض النووي الريبي لفيروس سى وسجلت نسبه حساسية ٩٨٪ وخصوصية ١٠٠٪. وانه لا توجد فروق ذات دلالة احصائية فى كميته الحمض النووي الريبي لفيروس سى بين عينه السيرم ونقطه الدم الجاف بين المرضى. اما بالنسبه للمستويات المختلفة من الحمل الفيروسي، فقد لوحظ عدم وجود فروق ذات دلالة احصائية بين العينتين عندما كان الحمل الفيروسي اقل او أكثر من ١٠٠٠٠٠ وحدة دولية.

وباختصار يمكن القول بان استخدام نقطة الدم الجافه لتضخيم الحمض النووي الريبي سى، طريقه موثوق بها، حساسة، رخيصة، سهله ومناسبة لمتابعه المرضى. ويمكن استخدامها فى الكشف عن فيروس سى لدى الاشخاص المعرضين للاصابه بصوره كبيره مما يزيد من امكانيه وسهوله التشخيص المبكر وبالتالي زيادة فرص الحصول على العلاج.