

## Simple and Reliable Method for Detection and Genotyping of Hepatitis C Virus RNA in Dried Blood Spots Stored at Room Temperature

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**We describe a simple, sensitive, and reproducible method for using whole blood collected onto filter paper (dried blood spots) for detection and genotyping of hepatitis C virus RNA that can be useful in large field studies, particularly in settings where collection, preparation, storage, and shipment of samples at controlled temperature can be difficult.**

Dried blood spots (DBS) have been used worldwide for the neonatal screening of congenital disorders (2, 8). Seroepidemiological studies have been conducted on DBS residual to neonatal screening to assess human immunodeficiency virus (HIV) prevalence among childbearing women (9, 10). Recently, several studies were focused on DBS for detecting drug resistance mutations (6) and for tracking global spreading of HIV type 1 subtypes (4) in proviral HIV DNA. However, RNA is notoriously less stable, and standardization of DBS for viral RNA detection would be of great benefit for application to large field studies, since DBS collection is easy, does not require skilled phlebotomists and expert technicians, and is suitable for storage and shipment to laboratory in settings where these issues are problematic. Recently, dried plasma spots and DBS have been used for HIV RNA detection and quantification, showing good correlation with titers obtained with conventional plasma samples (5, 11). However, these observations were limited to short storage at room temperature (11) or at 37°C (5), and a loss of viral titers occurred during storage.

For hepatitis C virus (HCV) RNA detection on dried spot samples, the available data are much less exhaustive. A complete match between frozen serum and dried plasma spots, though with a loss of titers after room temperature storage, has been observed (1).

This study was aimed at developing a simple, sensitive, and reproducible method for using DBS in HCV RNA detection and genotyping.

The study complied with all relevant national guidelines and institutional policies. Residual laboratory samples of EDTA-whole blood of 39 HCV antibody (Ab)-positive and 16 HCV Ab-negative patients, undergoing routine hematological controls, were used.

HCV Abs were determined by third-generation assay (Abbott Diagnostics). Among the Ab-positive patients, 34 had HCV RNA levels ranging between 9,640 and 5,100,000 IU/ml

(Amplicor HCV Monitor; Roche Molecular Systems Inc.), and 5 were HCV RNA negative (Versant HCV TMA; Bayer Diagnostic Inc.). The HCV genotype was known for eight patients (four had 1b; two had 2a/2c; one had 3a; and one had 4c/4d).

DBS were realized within 5 h from venipuncture by carefully spotting, in multiple replicates for each patient, 50 µl of EDTA-whole blood on SS grade 903 filter paper (Schleicher & Schuell Inc.). Two DBS from each patient were pooled and processed for each assay.

HCV RNA detection was performed with both in-house reverse transcriptase PCR (RT-PCR) and transcription-mediated amplification (TMA). For RT-PCR, RNA extraction was performed with Boom technology, utilizing silica-based RNA isolation (3), which was purchased from Organon Teknika. Specifically, DBS were cut, placed in 9 ml of lysis reagent, rocked 2 h at room temperature, and removed; supernatants were extracted according to the manufacturer's instructions. Ten microliters out of the 50-µl RNA eluate underwent the subsequent steps. Reverse transcription was performed with 50 U of Moloney murine leukemia virus RT (Gibco BRL, Life Technologies) in 20 µl. Ten microliters of cDNA was used in the nested PCR, according to reference 13. The amplified products were analyzed by agarose gel electrophoresis (amplicon size, 197 bp). TMA, provided by Bayer S.p.A. Divisione Diagnostici Italia, is a recently developed HCV RNA qualitative detection method, based on 5' untranslated region-targeted magnetic separation, followed by reverse transcription and T7 RNA polymerase-driven isothermal amplification (15). DBS from each patient were soaked in 500 µl of distilled water supplemented with 400 µl of Target Capture Reagent and were incubated at 60°C for 60 min. Then supernatants were decanted; the subsequent extraction and detection steps followed the standard TMA protocol.

A total of 158 DBS pairs were tested (124 from 34 HCV patients; 24 from 19 HCV-negative individuals). On the whole, with both TMA and RT-PCR all the 124 DBS pairs from positive patients tested positive, and all the 24 DBS pairs from the HCV RNA-negative patients tested negative (sensitivity >

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TABLE 1. Establishment of detection limit of HCV qualitative RNA assay (TMA) applied to DBS<sup>a</sup>

HCV RNA serum concn (in IU/ml)	Estimated input (in IU) of HCV RNA <sup>b</sup>	No. positive/no. tested	% Positive results
604,000	33,250	8/8	100
120,800	6,640	8/8	100
24,160	1,328	8/8	100
4,830	265	6/7	85.7
960	53	6/8	75
190	10.6	0/8	0
38	2.1	0/8	0
0	0	0/8	0

<sup>a</sup> Uncoagulated blood from a viremic patient (viral burden, 604,000 IU/ml) was diluted with fresh blood from an HCV-seronegative individual. Eight five-fold serial blood dilutions were spotted to form multiple replicate DBS that were extracted and assayed as described in the text.

<sup>b</sup> Estimated HCV RNA input in the DBS was determined by adjusting values for viremia in serum for the volume of blood spotted and for the relative hematocrit value.

99%; specificity > 95%). No genotype restriction was observed.

The detection limit was established by limiting dilution analysis. Specifically, blood samples from two viremic patients (viral burdens, 604,000 and 807,000 IU/ml) were serially diluted with blood from an HCV-seronegative individual. Seven five-

fold serial blood dilutions were spotted to form multiple replicate DBS strips. Four replicate pairs of each dilution (24 tests per run) were tested with TMA (after 1 and 5 weeks), giving a total of 48 tests performed, and with RT-PCR. The results, shown in Table 1, indicate that TMA was positive in 100, 85.7, and 75% of DBS containing, respectively, 1,328, 265, and 53 IU of HCV RNA. Similar sensitivity was observed with RT-PCR (not shown).

To evaluate the cross-contamination risk, a panel of DBS strips was prepared by spotting in close vicinity and, in alternate order, 15 blood samples from HCV RNA-positive patients and 15 blood samples from HCV Ab-negative individuals. Representative RT-PCR results are shown in Fig. 1, indicating a complete correspondence between expected and observed results. Similar results were obtained with TMA (not shown).

To test the stability of HCV RNA in DBS over time, a set of replicate DBS from 16 HCV RNA-positive patients was stored at room temperature and assayed at intervals of 2 to 4 weeks over an 11-month period. The results indicate that 100% positivity was preserved along the whole observation period.

To test the suitability of DBS for HCV genotyping, a replicate set of DBS from the eight patients with known genotype, stored at room temperature for 11 months, was extracted as for RT-PCR and genotyped by using the Line Probe assay (Bayer

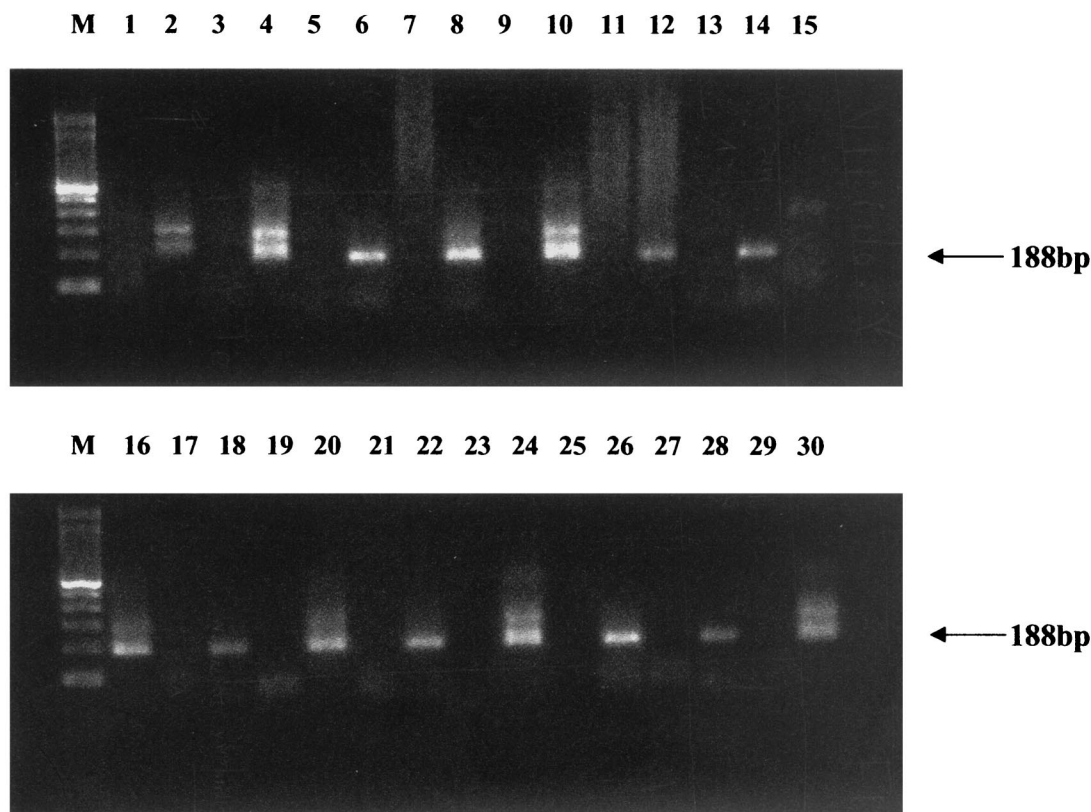


FIG. 1. Detection of HCV RNA in DBS from HCV-positive and -negative individuals. To control the risk of cross-contamination in using DBS for HCV RNA detection, DBS strips were prepared with blood from 30 different patients that was expected to be positive (uneven lines) or negative (even lines). The strips were spotted to realize a lattice of alternately HCV RNA-positive and -negative samples. DBS strips were wrapped in their individual paper covers and stored together in the same plastic bag for 1 week at room temperature before the assay. Representative results obtained with RT-PCR are shown.

Diagnostic Inc.). The genotype, determined in all tested DBS, was in agreement with that obtained on frozen serum samples.

On the whole, our findings show that DBS, stored at room temperature for prolonged periods, are suitable for HCV RNA detection and are also useful for HCV genotyping. The assessment of HCV genotype distribution at population level may help in defining the epidemiological dynamics of HCV infection (12, 14). Since HCV genotype may influence the response to antiviral treatment, severity of infection, and performance of diagnostic assays (7, 16), these aspects are particularly relevant in view of the possibility of using the method described here in large field studies and for application in settings where collection, centrifugation, storage, and shipment can be difficult, as is often the case in developing countries.

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