

## Hepatitis B Virus DNA Can be Amplified Directly From Dried Blood Spot on Filter Paper

<sup>1</sup>Naglaa Fathy Alhusseini, <sup>1</sup>Mamdouh Z. Abadeer and <sup>2</sup>Sheref M. El-Taher

<sup>1</sup>Department of Biochemistry,

<sup>2</sup>Department of Community Medicine,  
Faculty of Medicine, Benha University, Egypt

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### ABSTRACT

Hepatitis B Virus (HBV) infections represent a major public health problem because of the ability of HBV 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. They are easy to obtain, stable and can be transported to a reference laboratory at minimal cost. This study was to evaluate the feasibility of DBS samples for direct amplification of HBV DNA bypassing nucleic acid extraction. Results obtained from DBS samples were compared from those from plasma by routine molecular technique and also with those from whole blood. On the whole results for DBS, whole blood and plasma samples for HBV-DNA semi quantitative PCR monitoring, demonstrated very good agreement. This study also represents the first report in Egypt to evaluate the use of DBS to direct amplification of HBV-DNA and concluded that the use of DBS for direct amplification of HBV DNA without nucleic acid extraction was reliable, specific, sensitive, cheap and appropriate method to monitor the HBV infected patients.

**Keywords:** Hepatitis, HBV, DBS, Dried Blood Spot, PCR, Hepatitis B Carrier, CHB

### 1. INTRODUCTION

Hepatitis B Virus (HBV) infection is a problem in several regions of the world with limited resources Sumathi *et al.* (2010). (HBV) causes transient and chronic infections of the liver. Transient infections may produce serious illness and approximately 0.5% terminates with fatal, fulminate hepatitis. Chronic infections may also have serious consequences: nearly 25% terminate in untreatable liver cancer. Worldwide deaths from liver cancer caused by HBV infection probably exceed one million per year (Lira *et al.*, 2009; Viviana *et al.*, 2008)

The diagnosis and monitoring of HBV infection is generally based on the determination of serologic markers and viral load quantification; however,

molecular characteristics such as genotype and genetic variants are not used routinely (Jardi *et al.*, 2004).

Dried Blood Spot (DBS) can be prepared with a finger prick rather than with venipuncture, causing less discomfort to the donor. Furthermore, these samples need minimal storage space, cause little biohazard risk and are convenient for transportation. DBS have been widely used to screen genetic and metabolic disorders and to investigate polymorphisms in parasitic diseases. They are ideally suited for large-scale population screening studies, including molecular epidemiological research and blood bank procedures. For the study of Hepatitis B Virus (HBV), Dried Blood Spot (DBS) samples have been used for detecting Hepatitis B virus surface antigen (HBsAg) and antibody to hepatitis B core

antigen (Ohishi *et al.*, 2006). Recently, DBS samples allowed the development of a simple, sensitive and appropriate test for quantifying HBV DNA and studying HBV genetic variants (Lira *et al.*, 2009).

In spite of the urgent need for an affordable and easy sampling method for viral load testing and monitoring of chronic HBV infection, there is no report on the role of DBS in evaluation of patients infected with HBV in Egypt where the hepatitis is endemic and a very big health problem.

So this study was to evaluate the feasibility of DBS samples for direct amplification of HBV DNA bypassing nucleic acid extraction. Results obtained from DBS samples were compared from those from plasma by routine molecular technique and also with those from whole blood in blinded experiment using the same technique.

## 2. MATERIALS AND METHODS

This is a cross sectional study, included 50 hepatitis B surface antigen-positive (HBs-Ag) patients from whom attending the Molecular Biology and Biotechnology Unit, Faculty of Medicine, Benha University for monitoring of HBV markers and/or viral load, during February to September 2011. Ten HBsAg negative subjects were selected as negative controls.

### 2.1. Sampling

Three mL anticubital venous blood sample was collected from each subject and placed immediately into vacutainer tube containing EDTA. Fifty  $\mu$ L was taken by Pastier pipette and spotted on filter paper Watman 903 for preparation of DBS. It was air dried at room temperature and then placed in plastic locked bag. One mL from whole blood was putted into 1.5 Eppendorf tube. The remained blood (about 2 mL) was centrifuged at 3500 rpm for plasma separation. The three form of each sample (DBS, whole blood and plasma) were stored at  $-80^{\circ}\text{C}$  for further processing.

### 2.2. Serological assays for HBsAg, HBeAg and Anti-HBe

The HBV serological markers were done from plasma samples using ELISA Kit, DiaSorin S.P.A Italy, according to the manufacturer's instructions.

### 2.3. Extraction of Viral DNA from Plasma Sample

Viral DNA was extracted from each plasma sample using QIAamp Viral DNA Extraction Kit (Qiagen,

GmbH) according to manufacture instructions for automatic extraction in QIAcube extractor (Qiagen, GmbH). The extracted DNA concentration was confirmed through measurement by UV Spectrophotometer. Readings were taken at wave lengths of 260 and 280 nm., according to that reported by (Alhusseini *et al.*, 2011). Concentration of DNA sample was measured =  $50 \text{ ug mL}^{-1} \times A_{260} \times \text{dilution factor}$  (Wilfinger *et al.*, 1997).

### 2.4. Amplification of HBV DNA by PCR

In rapid cycler PCR (G-Storm Thermal cycler, England) 10  $\mu$ L from each sample of extracted DNA were used in single plex-PCR for HBV genomic DNA using PCR master mix kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions. Amplification was performed using the following primer sets provided by (Operon, inc Huntsville, Alabama Germany). Forward primer 5'-TCTGCCTAATCATCTCATG-3 'reverse primer 5'-GCCTCGTCGTCTAACAA-3'.

### 2.5. Direct Amplification of HBV-DNA on DBS and whole Blood Without Nucleic Acid Extraction

Using the same primers pair for HBV, direct amplification was done for each DBS and whole blood sample using KAPA blood PCR Kit (KAPA Bio system U.S.A). The reaction mix prepared using 2.5  $\mu$ L EDTA blood per 25  $\mu$ L rxn, or 3mm diameter disc from each DBS on filter paper using sterile punch. According to the manufacturer instructions the applied amplification program was  $95^{\circ}\text{C}$  for 5min. as initial denaturation then cycling for 40 cycle ( $95^{\circ}\text{C}$  30 Sec.,  $48^{\circ}\text{C}$  30 Sec. and  $72^{\circ}\text{C}$  1 min.). The final extension was  $72^{\circ}\text{C}$  for 2 min.

### 2.6. Post-PCR Processing and Analysis of the Amplified Products

After completion of the PCR program the obtained amplified products were centrifuged at 14,000 rpm speed to obtain the most compact pellet of organic debris (and facilitate recovery of the amplicon-containing supernatant).

The amplified DNA was analyzed by electrophoresis. About 10  $\mu$ L of each reaction mixture and 1000 Base Pair (BP) ladder (Molecular weight marker) was separated on 2% agarose gel containing  $0.3 \text{ ug mL}^{-1}$  of ethidium bromide. The bands were visualized using UV Tranilluminator (254 nm) and photographed using a digital camera 8 mega pixel. The image was transferred to be analyzed by computer software (Alpha In no Tech Gel Documentation System).

### 2.7. Statistical Analysis

Statistical analysis was undertaken using SPSS computer software (SPSS Version 16 for Microsoft Windows, SPSS Inc., Chicago) and the Microsoft office Excel 2007. Quantitative data are expressed in terms of mean, standard deviation and qualitative data were expressed in number and percent, appropriate statistical tests were used (ANOVA “f” test and correlation coefficient “r” test). ROC curve analysis to determine the diagnostic power of each test. Results were considered to be statistically significant at  $p < 0.05$ .

### 3. RESULTS

To validate the utility of DBS and method of amplification of viral DNA directly in the sample without extraction, Viral Load (VL) determination of matched samples from plasma. Whole blood and DBS were performed. Viral DNA was extracted from plasma samples only and amplified as routine HBV amplification methods. All selected patients were HBsAg positive but eight of them gave undetectable results in PCR for different three samples. The clinical and virological data of the all 50 HBsAg positive patients were described in **Table (1)**. There is no statistical significant difference in hepatitis B viral load between the three different samples among the patients (DBS, whole blood and plasma samples)  $p > 0.05$  **Table (2)**. Sensitivity and specificity for DBS samples and the direct amplification method are 100% as compared to negative control samples. It also 100% as compared to other two samples **Fig. (1 and 2)**. As regarded to differences between samples in different levels of viral

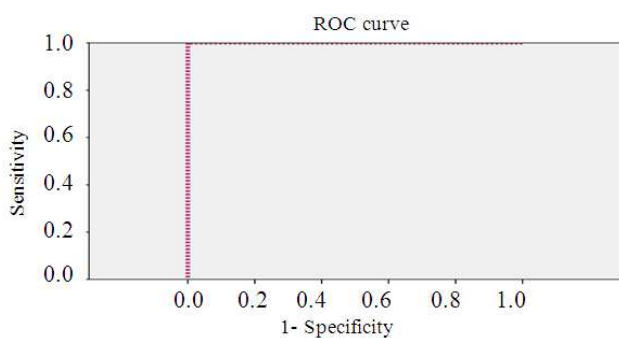
load, we demonstrate that there is no statistical significant different between the three types of samples when viral loads are less than and also more than 100000 IU mL<sup>-1</sup>, n. = 23, f = 0.4 and n. = 19, f = 1.1 respectively  $p > 0.05$ . The viral load amplified from DBS and whole blood samples are higher than that of plasma in case of low viremia  $< 100000$  IU mL<sup>-1</sup> **Fig. (3)**.

**Table 1.** Clinical and virological patients data

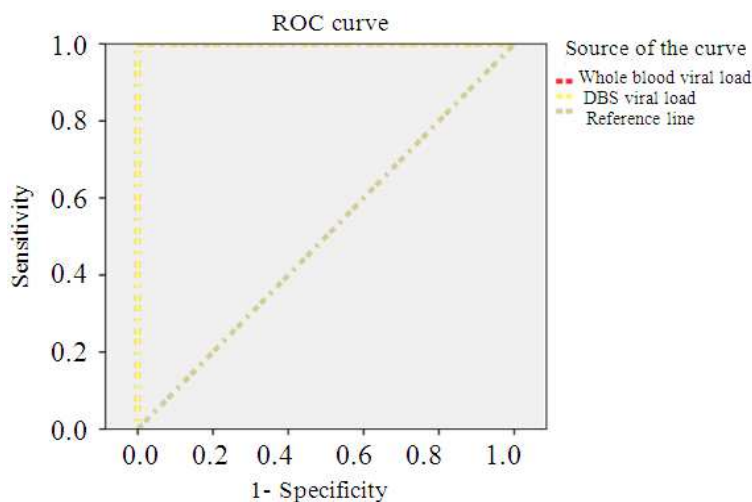
Sex: ♂:♀ %	32:18 64%: 36%
Age: / year Mean ± SD (rang)	30.58±9.57 (16-54)
HBsAg +Ve	6/50 12%
AntiHBe +Ve	4/50 8%

**Table 2.** Comparison of log10 I.U of HBV load between different samples

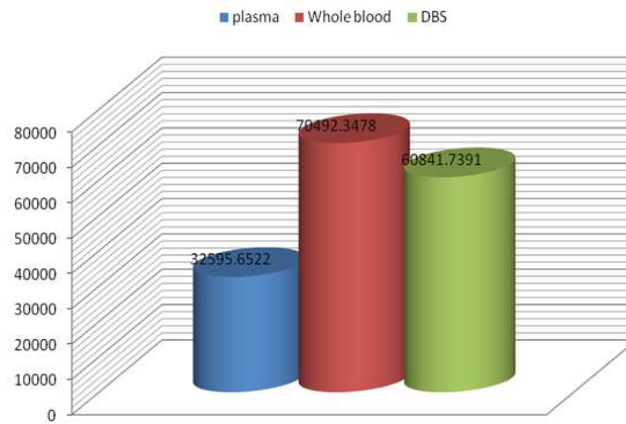
Sample type	Log10 of viral load Mean ± SD	F	P
Plasma	4.85±0.73		
Whole blood	4.87±0.67	1.04	> 0.05
DBS	4.66±0.76		



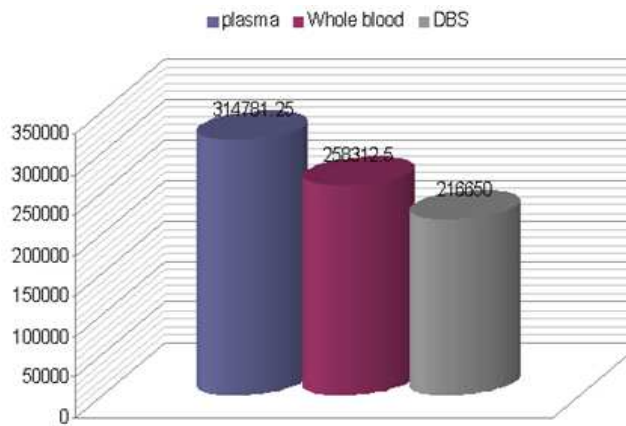
**Fig. 1.** ROC curve for DBS



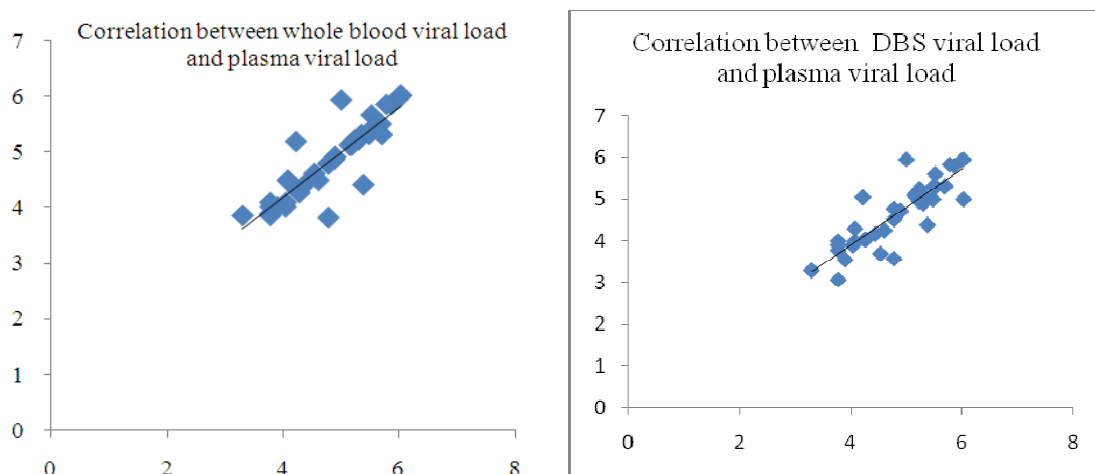
**Fig. 2.** ROC curve for both DBS and whole blood viral load : Area under the Curve for both viral load of whole blood and DBS is 1.000



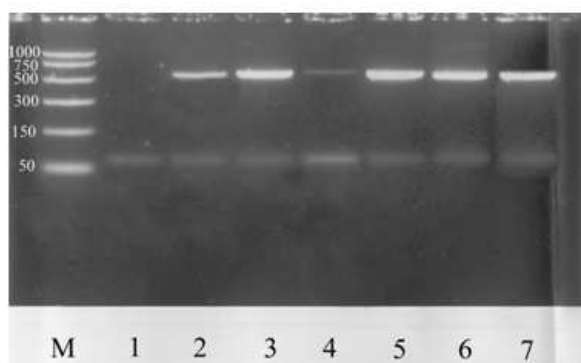
**Fig. 3.** Comparison between three methods when viral load is less than 100000 IU



**Fig. 4.** Comparison between three methods when viral load is more 100000 IU



**Fig. 5.** Correlation coefficient “r” between viral load of whole blood, DBS and those of plasma samples



**Fig. 6.** Gel electrophoresis of the amplified products of HBV in different three samples, M: 1000 bp marker. Lane 1; indicate negative control. Lanes 2, 3, 4: Indicate positive for HBV with low viral load in DBS, whole blood and plasma samples correspondingly. Lanes 5, 6, 7: Indicate positive HBV with high viral load in DBS, whole blood and plasma samples correspondingly

In contrast the viral load amplified from plasma samples are higher than those of both DBS and whole blood in case of moderate and high viremia  $> 100000\text{IU mL}^{-1}$  **Fig. (4)** There are significant positive correlations between viral load among plasma samples and those of DBS and whole blood samples  $r = .0.88$  and  $0.86$  respectively  $p < 0.001$  **Fig. (5)**. The amplified products of different samples and different viral loads were uploaded on gel electrophoresis and the variation of different bands appeared as seen in **(Fig 6)**.

#### 4. DISCUSSION

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 (Tuailon *et al.*, 2010). In addition this technique can reduce the cost of HBV testing by simplifying sample collection, processing (no centrifugation), storage and shipment. The value of DBS was for detection of HBV, HCV and HIV infection (Alter *et al.*, 2003) and (Ohishi *et al.*, 2006). Also other studies demonstrated the value of DBS for isolation and amplification of nucleic acid (RNA/DNA) of viral particles (Jardi *et al.*, 2004; Tuailon *et al.*, 2010).

Dried Blood Spot (DBS) sampling involves the collection of whole blood samples by heel or finger-prick which is then dropped onto a filter paper, is a convenient method for obtaining and handling clinical samples

intended for further analysis (Brambilla *et al.*, 2003). This type of sampling is useful in resource-limited environments especially in the developing countries where these procedures for obtaining and handling samples for laboratory analysis could possibly reduce costs. It is a simple sampling method which requires minimal training and the risk of injuries is eliminated as needles and syringes 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 (Lee *et al.*, 2011).

In this study we have modified a method for the detection of HBV DNA directly on DBS by using PCR methodology. The detection of HBV DNA is traditionally carried out by PCR amplification of DNA isolation from serum/plasma (Cheyrou *et al.*, 1991; Yokosuka *et al.*, 1991). 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 HBV DNA by using PCR methodology directly on DBS (Gupta *et al.*, 1992). This methods has actually bypass the veinipuncture and plasma/serum separation. Jardi *et al.* (2004) have assessed the DBS samples as an alternative to serum and found an important limitation of DBS. This limitation was the smaller DNA yield, that was confirmed when a significant factor where quantitative DNA analysis was concerned. They explained that may be related mainly with the dilution of the DBS samples inherent to the procedure of DBS preparation. Taylor *et al.* (2009) found that the precipitation methodology occurred during extraction may affect the detection level of viral load specially in cases of low viremia. In this study we overcome this limitation by bypass the extraction processing and amplify the DNA directly from DBS. A limitation that suspected to inhibit our amplification was in presence of heme in DBS which inhibit the PCR (Gupta *et al.*, 1992). In this study we use a high fidelity 2nd generation hot start polymerase enzyme that resist inhibitors (Woolf *et al.*, 2005).

Similar to many other studies prior to this DBS collection and processing were performed by controlled application of EDTA blood onto filter paper in laboratory conditions instead of whole blood collected directly from heel or finger-prick in a real-world situation (Uttayamakul *et al.*, 2005; Castro *et al.*, 2008; Kane *et al.*, 2008; Mbida *et al.*, 2009). Although the method of DBS collection onto the circles on the filter paper is easy and requires minimal training, the final appearances of the blood spots on the filter papers would

still require standardization. Few studies have been conducted so far regarding the effectiveness of this DBS collection method in the field, collecting samples via infant heel-prick. Once validated, this method of blood sample collection should be able to be performed in communities in remote areas, without the need for advanced equipment, expensive transportation or shipping, maintenance of cold chain, risks associated with the handling of potentially infected material and all the costs involved (Lee *et al.*, 2011).

## 5. CONCLUSION

This study concluded that the use of DBS for direct amplification of HBV DNA without nucleic acid extraction was reliable, specific, sensitive, cheap and appropriate method to monitor the HBV 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. This study also represents the first report in Egypt to evaluate the use of DBS to direct amplification of HBV-DNA by the second generation derived DNA polymerase using commercial kit available.

### 5.1. Recommendations

We recommended studying the use of DBS for monitoring hepatitis C virus infection in Egypt. Also we have to modify the amplification directly to DBS using Real time PCR.

## 6. ACKNOWLEDGEMENT

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