THE USE OF DRIED BLOOD SPOTS FOR MEASURING THE IMMUNITY AGAINST HBV: THE SIGNIFICANCE OF ANTIBODY DILUTION

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ABSTRACT

Background: Dried blood spots (DBS) have been used to study the prevalence of hepatitis B virus (HBV) infection in endemic areas and in high-risk groups. However, detection of HBV serological markers in DBS by ELISA assays has not yet been fully optimised. The aim of the present study is to evaluate the dilution level of anti-HBs when DBS cards are used as storage matrix implemented for ELISA.

Material and methods: Antibodies against hepatitis B surface antigen (anti-HBs) were detected by ELISA. The following specimens were examined: serum samples from 20 patients paired with 20 DBS; serum samples from 20 HBV-vaccinated healthcare workers paired with 20 dried serum spots (DSS); and four different dilutions of Immunovenin. Different elution protocols were used in order to study the problem with sample dilution.

Results: Specificity of 100% and sensitivity of 45% were established for DBS versus the "gold standard". Dilution of the eluted DBS/DSS samples was established and in some cases the measured anti-HBs titre dropped under 10 mIU/ml. Correlation was not observed between the positive initial anti-HBs serum titres and the obtained values of DBS/DSS testing. Also, 20- to 50-fold dilutions were measured for eluted DSS samples when testing Immunovenin. Increasing of the eluted sample concentration raised DSS anti-HBs titre.

Conclusions: In order to resolve the problem of dilution, it is necessary to validate different elution protocols because the small amount of sample in DBS showed lower titres.

KEYWORDS:

DBS, anti-HBs, HBV

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INTRODUCTION

Hepatitis B virus (HBV) infection represents a major health burden worldwide with 248 million people chronically infected in 2010 (1). In 2014, 22442 newly diagnosed HBV infection cases were reported from 30 EU Member States (2). In 2017, 249 cases of viral hepatitis B were reported from Bulgaria, an incidence of 3.51%$_{000}$, with 10 reported deaths (mortality of 0.14%$_{000}$, lethality of 4.02%). All reported cases were laboratory confirmed (3). Up to 90% of HBV transmissions can be prevented with administration of immunoglobulin within 48 hours after birth or with implementation of HBV vaccine series, beginning at birth and completed within 12 months (4). In 1992, the World Health Organisation recommended all countries to include HBV vaccination in the universal childhood vaccination program. Since 1991, an obligatory vaccination has been implemented for all newborns in Bulgaria (5).

Serological markers used in the diagnosis of HBV infection are HBsAg, anti-HBs, HBeAg, anti-HBe and anti-HBc IgM/IgG (6). Anti-HBs is a neutralising antibody and serves as a marker for long-term immunity. In vaccinated patients, anti-HBs is the only serological marker detected in serum. In the case of past HBV infection these antibodies appear with anti-HBc IgG. Occasionally, the simultaneous appearance of HBsAg and anti-HBs has been reported in HBsAg-positive patients (7). Anti-HBs becomes detectable during convalescence after the disappearance of HBsAg in patients who do not progress to chronic infection. The presence

of anti-HBs following acute infection generally indicates recovery and immunity to re-infection (8). At the same time rapid anamnestic increase of anti-HBs titres in HBV-vaccinated persons is a strong evidence of resent HBV infection (9).

Dried blood samples (DBS) on filter paper were originally used for the measurement of phenylalanine in newborns for detection of phenylketonuria (10). DBS or Dry Plasma Spot (DPS) is a recent technology used for collection, storing and transportation of blood samples. DBS provides a number of advantages over whole blood, serum or plasma sample collection. Sampling is simple and minimally invasive versus whole blood venipuncture. The biggest advantage of DBS is shipping and storage requirements. Samples are small, there are no requirements for cold chain and samples can be transported in standard post envelop (11). Only eight studies have evaluated the use of DBS for diagnosis of hepatitis viruses. Their potential was estimated for HCV, HAV and HBV serology (12). DBS have been used to study the prevalence of HBV infection in endemic areas and in high-risk groups. However, detection of HBV serological markers in DBS by immune-linked immunosorbent assays (ELISA) has not yet been fully optimised (13), especially anti-HBs. The aim of the present study is to evaluate the dilution level of anti-HBs when DBS is used as storage matrix implemented for ELISA.

MATERIAL AND METHODS

Patients and specimens: A total of 40 persons with two types of clinical material were tested: serum samples from 20 patients paired with 20 DBS (study group I); serum samples from 20 HBV-vaccinated healthcare workers paired with 20 dried serum spot samples (DSS) (study group II). The specimens were collected according to the requirements of the working program from research project Contract № DM 03/1,12.12.2016 funded by the National Science Fund, Bulgaria. The blood was collected by venipuncture and after centrifugation at 4000 rpm for 10 min the serum was separated, aliquoted and frozen at -20°C until analysed. In the case of study group I, 150 µl fresh blood were spotted on filter paper cards (HiMedia`s InstaDNA TM Cards) before centrifugation. For study group II, 150 µl serum sample was spotted on the cards. The filter cards were labelled appropriately, air dried at room temperature for 1 hour and stored individually in zip-locked bags with desiccant at 4°C for 1 month. Additionally, undiluted, 2-fold and 10-fold diluted Immunovenin intact 5% IgG (Bul Bio-NCIPD, Bulgaria) was applied on the filter cards and prepared following the same protocol. The protocol design and the study were performed in the National Reference Laboratory (NRL) ”Hepatitis viruses” at the National Centre of Infectious and Parasitic Diseases (NCIPD), Sofia, Bulgaria.

Elution of DBS: A 6-mm diameter disc was punched from each filter paper. One or two discs were incubated at room temperature in 200 µl or 500 µl elution buffer (PBS with 0.05% Tween) for 1 hour with agitation on a laboratory shaker. After that samples were incubated overnight at 4°C at rest. On the next day the samples were tempered and centrifuged at 10 000 rpm for 2 min according to the method by Mössner BK, et al (14). The filter paper discs were removed and DBS/DSS eluates were tested with anti-HBs ELISA.

Detection of anti-HBs by ELISA: Quantitative ELISA assays were performed and interpreted according to the manufacturer’s instructions (HBsAb ELISA, Dia.Pro, Italy). The amount of antibodies was quantitatively defined by means of standard curve calibrated against the WHO reference preparation. The ELISA assay had 98.9% diagnostic specificity and 100% diagnostic sensitivity. Samples with results of ≥10 mIU/ml were considered positive. All samples included in the study were retested in duplicate.

Statistical Analysis: All statistical calculations were performed using the Analysis ToolPak of Microsoft Office Excel 2010. For the statistical analysis we used relative percentages (%), correlation analysis, graphical and table analysis.

RESULTS

Detection of anti-HBs in serum and in DBS/DSS

Firstly, 40 serum samples were tested for the presence of anti-HBs. Half of the samples were from patients with different liver disorders and unknown anti-HBs status (study group I), and

the other 20 samples were from healthcare workers vaccinated against HBV (study group II). The median age of the patients was 34 ± 14 years and for the healthcare workers was – 45 ± 13 years. A total of 31 serum samples (78%, 95% CI: 65.16÷90.84) were anti-HBs-positive, with minimal antibody titre of 12 mIU/ml and maximal of 254 mIU/ml. Of these 31 positive samples, 11 were from study group I and 20 from study group II (Table 1). Next, the paired DBS samples of study group I and the DSS samples of study group II were tested for anti-HBs. For both groups elution was carried out with a 6-mm disc incubated in 500 µl elution buffer. Positive results with anti-HBs titre of >10 mIU/ml were found in 10 (25%, 95% CI: 11.58÷38.42) of all 40 filter card samples (DBS and DSS) with 5 anti-HBs-positive results in each of the study groups, respectively. The established sensitivity for both types of matrices was under 50%. The specificity of DBS was 100%. It was not possible to establish the specificity for DSS as all initially tested serum samples were anti-HBs-positive.

Table 1. Sensitivity and specificity of serological testing by HBsAb ELISA (Dia.Pro) of DBS/DSS compared with serum samples.

	Serum, n		DBS / DSS, n		Sensitivity [%]	Specificity [%]	PPV [%]	NPV [%]
	negative	positive	negative	positive				
Study group I	9	11	15	5	45	100	100	60
Study group II	0	20	15	5	33	NA	100	NA

Legend: PPV – positive predictive value; NPV – negative predictive value; NA – not applicable.

DBS/DSS were measured as anti-HBs-positive when the initial serum anti-HBs titre was over 200 mIU/ml (Table 2). At the same time, negative anti-HBs titres (<10 mIU/ml) were measured for the initial serum samples with anti-HBs titres between 225mIU/ml and 12 mIU/ml for DBS and between 226 mIU/ml and 20 mIU/ml for DSS.

Table 2. DBS/DSS anti-HBs results according to the initial serum titre.

Serum anti-HBs [mIU/ml]	DBS (n=20)		DSS (n=20)	
	Positive, n	Negative, n	Positive, n	Negative, n
10 - 50	0	3	0	3
50 - 100	0	1	0	2
100 - 150	0	1	0	5
150 - 200	0	0	0	2
200 - 250	5	1	5	3

Detection of anti-HBs in Immunovenin intact 5% IgG and in discs punched from filter cards

The possible dilutions of the samples were calculated before DBS/DSS testing. By following the designed protocol, 150 µl of the tested sample were dropped on HiMedia`s InstaDNA TM filter cards. When 6-mm discs were punched the sample volume decreased to approximately 9 µl (8.64µl). It was calculated that when one 6-mm disc (1 × 6 mm) was eluted in 500 µl elution buffer the sample is diluted 58-fold; in the case of two discs (2 × 6 mm) the dilution was 29-fold. In order to decrease the dilution, the design of the working protocol was modified and the discs

were eluted in 200 µl buffer with estimated 23- and 12-fold dilution, respectively.

The measured anti-HBs titres of the initial Immunovenin intact 5% IgG samples were 1440 mIU/ml, 880 mIU/ml, 1100 mIU/ml and 720 mIU/ml, respectively. For filter card samples anti-HBs decreased to 56 mIU/ml, 44 mIU/ml, 23 mIU/ml and 26 mIU/ml, respectively, when two 6-mm discs were eluted; and to 33 mIU/ml, 19 mIU/ml, 9 mIU/ml and 7 mIU/ml – when one 6-mm disc was eluted. As can be seen, anti-HBs titres obtained by the DBS collection method were decreased to extremely low levels – under10 mIU/ml (Fig. 1). Comparison of the results for the initial sample and for the two 6-mm discs demonstrated dilution ranging from 20- to 50-fold.

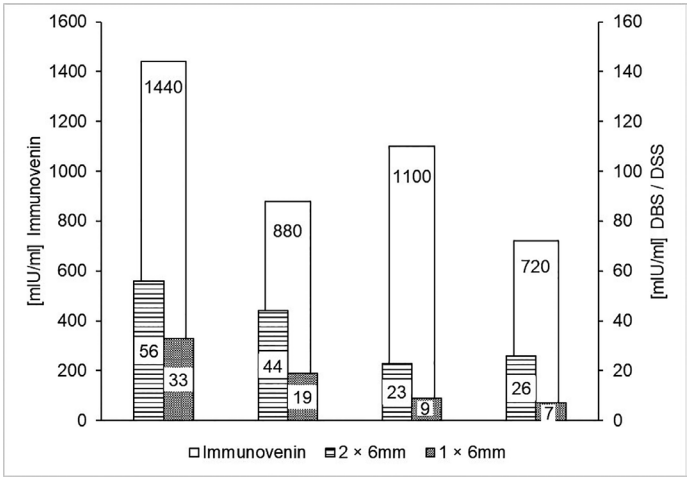

Figure 1. Measured anti-HBs [mIU/ml] titer in initial samples Immunovenin intact 5%IgG and paired DBS/DSS samples

Legend: 2 × 6mm – two 6 mm punched discs were eluted in 200 µl elution buffer; 1 × 6mm - one 6 mm punched disc was eluted in 200 µl elution buffer. Anti-HBs titer of four series Immunovenin intact 5%IgG with different initial concentrations and their paired 2 × 6mm and 1 × 6mm filter paper discs were tested.

The positive correlation for anti-HBs antibody titres obtained with two 6-mm discs and one 6-mm disc samples is shown in Fig. 2 with a correlation coefficient of 0.97. As can be seen, when the sample concentration increased from 9 µl (one 6-mm disc) to 18 µl (two 6-mm discs) the anti-HBs titre almost doubled.

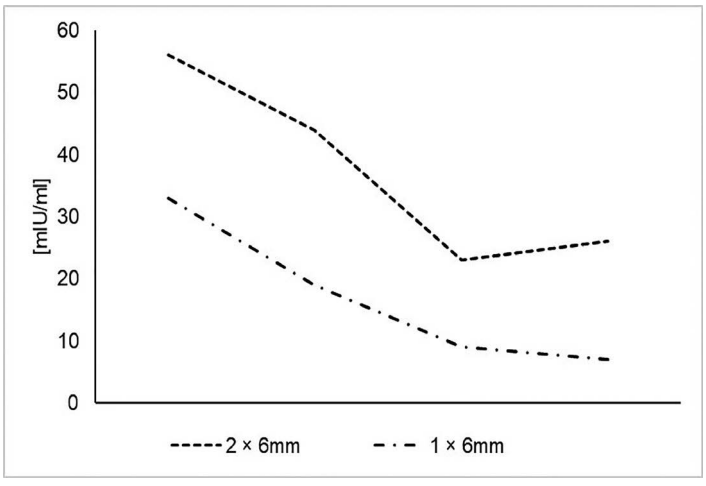

Figure 2. Correlation between the number of eluted discs and anti-HBs titre.

Legend: 2 × 6 mm – two 6-mm punched discs were eluted in 200 µl elution buffer; 1 × 6 mm – one 6-mm punched disc was eluted in 200 µl elution buffer.

DISCUSSION

In many aspects DBS is a convenient method for clinical sample collection (15). Although they can be used as sample storage matrix in HAV and HCV diagnosis and detection (16), their relevance in detection of some HBV markers, such as anti-HBc and anti-HBs, is limited (14). Although this study established 100% specificity for DBS, the sensitivity is too low – 45%. The low sensitivity for anti-HBs in DBS versus plasma was observed even when using automated diagnostic platform (17). There are two possible explanations: first, the number of tested samples in the present study is too small; and second, the different starting

titres of the samples can be the reason for the different dilution factor with elution (14). During the study it was established that the eluted DBS/DSS samples were diluted 12- to 23-fold and in some cases the measured anti-HBs titre dropped under 10 mIU/ml. Mössner, et al observed a similar correlation showing decreasing anti-HBs titre when adding elution buffer (14). Similar dilution was established when the initial sample was Immunovenin intact 5% IgG with eliminating the influence of hematocrit and other host factors on sample absorption and spread across the filter paper. As it is already known, hematocrit has an impact on different variables when using the DBS protocol (18). We established a positive correlation when the volume of the eluted sample is increased by using two instead of one disc. False negative results were eliminated. To resolve the problem of dilution, it is necessary to validate different elution protocols because the small amount of sample in DBS led to lower titres in comparison with the "gold standard" for anti-HBs detection.

CONCLUSIONS

DBS/DSS can serve as an alternative option in anti-HBs screening, epidemiological studies and sample storage. The test shows high specificity but additional studies are needed to overcome the problem with anti-HBs dilution after elution.

Competing Interest

The authors have declared that they have no conflicts of interest.

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REFERENCES

1. Schweitzer A, Horn J, Mikolajczyk RT, Krause G, Ott JJ. *Estimations of worldwide prevalence of chronic hepatitis B virus infection: a systematic review of data published between 1965 and 2013*. Lancet. 2015; 386(10003):1546–1555.
2. European Centre for Disease Prevention and Control. Hepatitis B Surveillance in Europe – 2014. Stockholm: ECDC, 2016.
3. Kurchatova A, Vladimirova N, Minkova A, Kamenov G, Stoitsova S, Parmakova K. *Acute infectious diseases in Bulgaria in 2017 (Key Epidemiological Indicators)*. https://www.ncipd.org/index.php?option=com_docman&view=list&slug=analysis-2017&Itemid=1127&lang=bg
4. Børresen ML, Koch A, Biggar RJ, Ladefoged K, Melbye M, Wohlfahrt J, Krause TG. *Effectiveness of the targeted hepatitis B vaccination program in Greenland*. Am J Public Health. 2012; 102(2):277-284.
5. Todorova TT, Tsankova G, Lodozova N. *Hepatitis B infection in eastern regions of Bulgaria*. J IMAB. 2016; 22(1):1033-1035.
6. Golkocheva-Markova E, Alexiev I, Teoharov P. *Relation between hepatitis B surface antigen kinetic and viral replication in patients with resolved hepatitis B virus infection*. Probl Infect Parasit Dis. 2013; 41(2):24-28.
7. Song JE, Kim DY. *Diagnosis of hepatitis B*. Ann Transl Med. 2016; 4(18):338.
8. World Health Organization. Hepatitis B: Serological and clinical patterns of acute or chronic HBV infections. WHO 2002. http://www.who.int/emcdocuments/hepatitis/docs/whocdscsrlyo20022/disease/serological_clinical_pattern.html
9. Su TH, Chen PJ. *Emerging hepatitis B virus infection in vaccinated populations: a rising concern?* Emerg Microbes Infect. 2012; 1(9):e27.
10. Guthrie R, Susi A. *A Simple Phenylalanine Method for Detecting Phenylketonuria in Large Populations of Newborn Infants*. Pediatrics. 1963; 32:338-343.
11. Chace DH, De Jesús VR, Spitzer AR. *Clinical chemistry and dried blood spots: increasing laboratory utilization by improved understanding of quantitative challenges*. Bioanalysis. 2014; 6(21):2791-2794.
12. Smit PW, Elliott I, Peeling RW, Mabey D, Newton PN. *An overview of the clinical use of filter paper in the diagnosis of tropical diseases*. Am J Trop Med Hyg. 2014; 90(2):195-210.
13. Marques B, Brandão C, Silva E, Marques V, Villela-Nogueira C, Do ÓK, de Paula M, Lewis-Ximenez L, Lampe E, Villar L. *Dried blood spot samples: Optimization of commercial EIAs for hepatitis C antibody detection and stability under different storage conditions*. J Med Virol. 2012; 84:1600-1607.
14. Mössner BK, Staugaard B, Jensen J, Lillevang ST, Christensen PB, Holm DK. *Dried blood spots, valid screening for viral hepatitis and human immunodeficiency virus in real-life.* World J Gastroenterol. 2016; 22(33):7604-7612.
15. Krumova S, Golkocheva-Markova E, Pavlova A, Angelova S, Georgieva I, Genova-Kalou. P. *Detection of measles and rubella antibodies in dried blood spots*. Probl Infect Parasit Dis. 2018; 46(2):20-24.
16. Smit PW, Elliott I, Peeling RW, Mabey D, Newton PN. *An overview of the clinical use of filter paper in the diagnosis of tropical diseases.* Am J Trop Med Hyg. 2014; 90(2):195-210.
17. Ross RS, Stambouli O, Grüner N, Marcus U, Cai W, Zhang W, Zimmermann R, Roggendorf M. *Detection of infections with hepatitis B virus, hepatitis C virus, and human immunodeficiency virus by analyses of dried blood spots--performance characteristics of the ARCHITECT system and two commercial assays for nucleic acid amplification*. Virol J. 2013; 10:72.
18. Lim MD. *Dried Blood Spots for Global Health Diagnostics and Surveillance: Opportunities and Challenges*. Am J Trop Med Hyg. 2018; 99(2):256-265.