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Short communication

Combined use of dried blood spot and rapid molecular systems: A robust solution to monitor hepatitis B virus infection with potential for resource-limited countries

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ABSTRACT

Chronic hepatitis B (CHB) particularly affects resource-limited countries. CHB management in these areas faces many obstacles for optimal care of patients, including poor access to HBV-DNA quantification, a key marker. This study aims to evaluate the quantification of HBV-DNA on dried blood spots (DBS) using rapid, standardized and fully automated on-demand systems. After a simple and rapid DBS elution protocol, HBV-DNA was simply and accurately quantified on this matrix using two different systems. Limit of quantification was estimated at 400 IU/mL. DBS and plasma HBV-DNA quantification provided comparable results. HBV-DNA stability for up to one month was demonstrated on DBS stored at room temperature, a condition compatible for preservation or transport before analysis. The combined use of DBS and commercially available automated molecular on-demand systems for HBV-DNA quantification could represent a reliable alternative in resource-limited countries to reach remote populations. The good sensitivity of this approach makes it attractive for mother-to-child transmission prevention, treatment decision and follow-up. Costs can be limited if such systems are also validated for other molecular markers.

Despite an effective vaccine, over 257 million people are chronically infected with the hepatitis B virus (HBV) worldwide. Resource-limited countries, particularly in African and Western Pacific regions, are exposed to specific situations regarding HBV infection: high prevalence, insufficient vaccination coverage and limited access to prevention, screening, monitoring and treatment. The treatment guidelines for the management of chronic hepatitis B (CHB) take into account clinical and biological criteria, ideally including HBV-DNA measurement in plasma World Health Organization, 2017. Unfortunately, resource-limited countries have currently limited access to this key marker in diagnosing and monitoring HBV infection. Dried blood spots (DBS) seem a practical alternative to plasma, the gold standard matrix. Several previous studies comparing HBV viral loads (VL) in plasma and DBS have used various methodologies, highlighting a large heterogeneity in DBS preparation and elution protocols and making comparison between each study difficult. Indeed, samples consisted of either fingertip or venipuncture, collected blood volume ranged from 20 to 100 µL, number of tested spots of different diameters varied and the elution processes used diverse buffers with or without a heating step. Likewise, these publications report various nucleic acid extraction processes and amplification strategies based on in-house or commercial molecular assays. Most of these methods have shown a significant loss of HBV-DNA quantification in DBS compared to plasma, from -1.59 to -0.59 $\log_{10}$ IU/mL, suggesting a poor yield of these protocols Lange et al., 2017. New systems for VL quantification, integrating sample loading, nucleic acid extraction, reaction preparation, real-time PCR and result interpretation are now available worldwide. These random access systems allow continuous and homogeneous processing of samples and reduce both hands-on and turnaround times. This study aims to evaluate the quantification of HBV-DNA on DBS using rapid, fully automated, on-demand systems Uceda et al., 2019.

HBV-free whole blood (WB) was spiked with HBV positive plasmas and serial 1:10 dilutions were performed in HBV-free blood to obtain an HBV-DNA range of 0.26–8.74 log IU/mL. DBS were prepared by deposition of 50 µL per spot of each dilution, either on a five-spots Whatman® protein saver card (Sigma-Aldrich, Copenhagen, Denmark) or on a pre-cut Munktell TFN card (Ahlstrom-Munksjö, Helsinki, Finland). Eluates were obtained by incubation of 2 spots (100 µL of WB)

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into 2 mL of phosphate buffered saline (PBS) under constant agitation for 2 h at room temperature (RT).

HBV-DNA was quantified using first DxN VERIS Molecular Diagnostics System (Beckman Coulter, Villepinte, France) and then Xpert GeneXpert System (Cepheid, Maurens-Scopont, France) HBV VL assays. Both assays lower limit of quantification (LLQ) on plasma samples is 10 IU/mL. Because none of these two assays includes a dedicated DBS protocol, the DBS eluates were analysed as recommended for plasma. Raw DBS-VL were multiplied by 40 to take into account the elution of two spots corresponding to 100 µL of WB, *i.e.* 50 µL of plasma by assuming a haematocrit value of 50 %, in 2 mL of PBS. As plasma and eluted DBS were then processed in a similar manner, no further correction factor was required to obtain the final result. Thus, the theoretical LLQ on DBS was calculated at 400 IU/mL (2.60 $\log_{10}$ IU/mL).

R^2 (Pearson correlation squared) was used to assess the correlation strength between quantification methods or between matrices. Bland-Altman analysis was used to document the agreement and the average difference between both quantitative methods or between both matrices. Statistical analyses were performed using GraphPad Software (Version 5.03, GraphPad Software, Inc).

A proof of concept emerged from a preliminary work performed on DxN VERIS showing that HBV-DNA measured on Whatman® protein saver card DBS, stored for 12 days at −80 °C was highly correlated to values obtained on plasma ($R^2 = 0.99$; $P < 0.0001$, data not shown). The Bland-Altman analysis indicated a mean difference measurement (DBS-plasma) of +0.31 $\log_{10}$ IU/mL in favour of DBS. However, false positive results suggested potential specificity concerns that were attributed to possible cross-sample contamination at the step of DBS puncture, despite thorough decontamination of the hole-punch apparatus between successive punches. Indeed, two out of the 6 negative controls, consisting of blank spots obtained by multiple punching of a clean unused card between each dilution, generated a low positive signal regardless of the virus load applied onto the previous spot. Robust quantification of HBV-DNA on DBS was then confirmed on precut Munktell TFN cards stored up to 14 days at RT, a condition closer to real situations. The contamination observed related to the DBS cutting system is concerning and has not been often reported for HBV. With high replication values in hepatitis B chronic carriers, this is certainly a preoccupying matter. A solution to avoid this major issue may solely be to use precut spots. This approach avoids using a puncher, the likely source of cross-contamination, thereby leading to better specificity. A similar difference measurement of +0.09 $\log_{10}$ IU/mL in favour of DBS between the two matrices (DBS-plasma) was found. Stability of HBV viral load on DBS was assessed by analysis with the VERIS HBV assay of DBS obtained from three patient's samples corresponding to 3 different viral loads (2.11, 3.14 and 5.50 $\log_{10}$ IU/mL) (Table 1). No clinically significant difference was found between the DBS analysed at 7 or 14 days and stored at room temperature or at −80 °C. These encouraging results prompted us to test the same approach using the simple worldwide available GeneXpert integrated system (Uceda et al., 2019).

In order to first compare HBV-DNA quantification with both Xpert and VERIS assays, ten HBV-infected patient plasmas were analysed in parallel on both systems. Obtained HBV-DNA loads ranged from 1.73 to 8.51 and 1.89–8.73 $\log_{10}$ IU/mL on the VERIS and the Xpert, respectively. One of the lowest plasma VL quantified by VERIS at 1.74 $\log_{10}$ IU/mL was not detected by Xpert. The Bland-Altman analysis showed a mean difference between the two assays (Xpert-VERIS) of +0.28 $\log_{10}$ IU/mL in favour of the Xpert assay. The extraction process may largely influence one method lower limit of detection. For all-in-one approaches such as Xpert cartridge or VERIS, each step (extraction and amplification) cannot be individualized. It is therefore difficult to analyze the specific reason why one assay would perform better than the other. Although 0.28 $\log_{10}$ IU/mL cannot be considered significant, this difference could potentially be attributable to the extraction protocol.

Then, to compare DBS *vs* plasma HBV-DNA quantification using the Xpert assay, eight paired plasma and DBS (Munktell TFN card), these last ones stored for 7 days at RT and obtained from positive sample serial dilutions were analysed with the Xpert HBV VL kit. One DBS corresponding to a theoretical plasma VL of 0.69 $\log_{10}$ IU/mL gave a positive signal (detected < 10 IU/mL without correction factor) below the theoretical LLQ on DBS (< 2.60 $\log_{10}$ IU/mL) corresponding to the claimed LLQ times 40, to take into account the DBS dilution factor. Linear regression performed on all dilution values showed a strong correlation between the two matrices ($R^2 = 0.99$; $P < 0.0001$) (Fig. 1A). The Bland-Altman analysis indicated a mean difference between the two matrices (DBS-plasma) equal to 0.07 $\log_{10}$ IU/mL. All quantitative results were within the limits of agreement (mean ± 2SD: -0.35 to 0.49 $\log_{10}$ IU/mL) (Fig. 1B). Quantification of HBV DNA from DBS stored for 31 days at RT did not differ whatever the VL level, with a mean difference d31-d7 = 0.07 $\log_{10}$ IU/mL (Table 1).

There is a need for more trustworthy, standardized methods for specimen preparation, storage and processing to provide reliable HBV-DNA quantification from DBS. Our study confirms previous results obtained on the DxN VERIS system in our lab for HBV-DNA quantification from DBS. After a simple and rapid elution protocol, HBV-DNA could accurately be quantified on DBS with the fully automated Xpert HBV VL assay. Interestingly, despite a lower limit of quantification estimated around 400 IU/mL on DBS and inherent to the sample and elution volumes, no significant loss of HBV-DNA quantification between DBS and plasma was observed contrary to previous published studies (Lange et al., 2017). This interesting point could be explained by the limited pre-analytical steps and the use of optimised fully automated

Table 1
Effect of DBS storage conditions and durations: Quantification of HBV-DNA on plasma and DBS on Munktell TFN paper after 7, 14 or 31 days under different storage conditions.

Sample	Sample dilution	HBV Assay	HBV-DNA Plasma $\log_{10}$ IU/mL)	HBV-DNA DBS ($\log_{10}$ IU/mL)				
				Room temperature			Frozen (−80 °C)	
				Storage time (day)				
				7	14	31	7	14
# 1	**No**	**VERIS**	2.11	3.25	3.26	NT	3.28	3.30
# 2	**No**	**VERIS**	3.14	3.79	3.93	NT	3.81	3.88
# 3	**No**	**VERIS**	5.50	5.41	5.43	NT	5.47	5.50
# 4	**No**	**Xpert**	7.55	7.49	NT	7.54	NT	NT
	$\mathbf{10^{-2}}$	**Xpert**	5.55	5.51	NT	5.51	NT	NT
	$\mathbf{10^{-3}}$	**Xpert**	4.68	4.42	NT	4.46	NT	NT
	$\mathbf{10^{-4}}$	**Xpert**	3.75	3.64	NT	3.69	NT	NT
	$\mathbf{10^{-5}}$	**Xpert**	2.55	2.75	NT	2.83	NT	NT

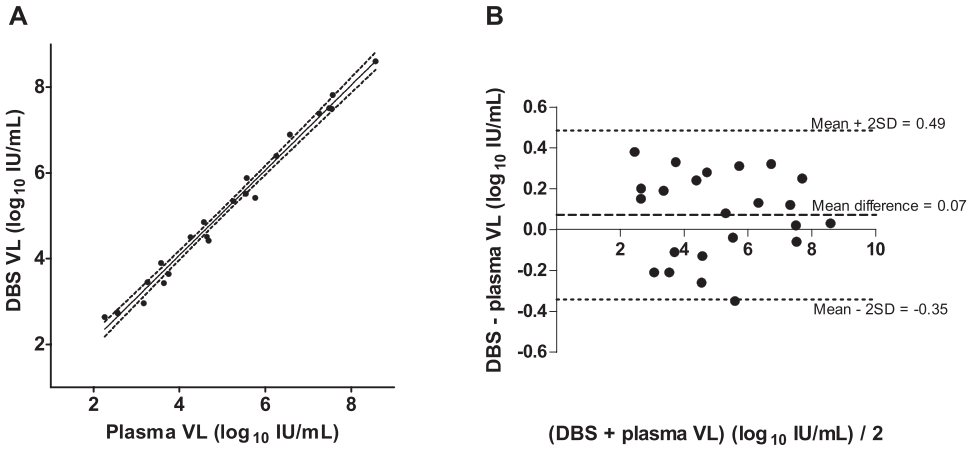

Fig. 1. Comparison of HBV-DNA quantification by Xpert HBV Viral Load assay on DBS and plasma. (A) Linear regression, (B) Bland-Altman analysis. VL, viral load.

systems. Indeed, the main works published on HBV-VL measurement from DBS report an under-quantification of HBV-DNA between -1.59 log IU/mL to -0.59 log IU/mL compared to plasma, whatever the processing analytical steps and the in-house or commercial used protocols. In addition, our study confirms HBV-DNA stability on DBS stored at RT for up to 31 days (Jackson et al., 2019). These results, while promising, certainly need confirmation on larger studies.

The combined use of DBS on an automated molecular test such as the Xpert HBV assay may sound contradictory since they both represent alternative solutions to follow remote HBV infected patients with no access to specialized laboratories. However, in a study assessing the use of GeneXpert systems for different markers, Ndlovu and colleagues pointed out that the lack of efficient blood sample transport networks increases the need for technologies such as point of care (POC) testing that can be performed on DBS samples (Ndlovu et al., 2018). Cepheid through the HIV-1 Qual Assay performed on the GeneXpert has already developed a test suitable for the use of DBS, however such approach is not available for HBV-DNA. Performing HBV-DNA quantification on a worldwide established system could drastically decreases the turn-around time compared to current methods requiring the shipment of DBS to larger cities, other countries and sometimes even other continents for analysis by an automated or low-cost PCR. Shortening the time to deliver results may also prove beneficial to rapidly initiate maternal antiviral prophylaxis in mother with high replication level, therefore limiting new cases of mother-to-child transmission of HBV or to identify and follow-up patients who need treatment (Society for Maternal-Fetal Medicine (SMFM) et al., 2016). In the proposed concept of the study, the Cepheid assay should not be perceived as a POC test but rather as a well-calibrated unitary system that can be used in remote areas and for samples shipped in suboptimal conditions in low-income countries. Our approach should certainly not be perceived as what is done for hepatitis C with the Xpert HCV Viral Load Finger-Stick solution where a rapid turn-around-time is useful to avoid loss of care (test and treat concept). For this reason, we do not believe the DBS elution time should be seen as a limiting factor. Indeed, the turn-around-time may reach 4 h following our protocol but shorter elution times could be tested. We believe Cepheid should be able to provide soon a finger-stick solution for HepB even though a rapid access to the result is not as critical as for HepC.

Using DBS and an automated molecular system opens the door to the possibility of a combined follow-up of HIV, HBV and HCV for the same patient if required. It can particularly be relevant in Sub-Saharan Africa where the burden of HBV co-infection among people living with HIV has been estimated at 2.6 million people (Platt et al., 2019).

To our knowledge, there has been no report describing the combined use of DBS and rapid, fully automated molecular system for HBV-DNA quantification. Such strategy could overcome encountered difficulties in resource-limited countries, where easy-to-use solutions are sometimes already implemented but not attainable to remote patients. In this proof of concept study, we mostly had access to artificially spiked samples. These encouraging results may help other investigators in the field to confirm and strengthen our findings on clinical samples and to validate the relevance of such approach for HBV-infected patient care.

Credit author statement

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CRediT authorship contribution statement

Pauline Bargain: Methodology, Validation, Formal analysis, Investigation, Resources, Writing - original draft, Writing - review & editing, Visualization. **Christopher Heslan:** Methodology, Validation, Investigation, Resources, Visualization. **Vincent Thibault:** Conceptualization, Methodology, Validation, Formal analysis, Investigation, Resources, Data curation, Writing - original draft, Writing - review & editing, Visualization, Supervision. **Charlotte Pronier:** Conceptualization, Methodology, Validation, Formal analysis, Investigation, Resources, Data curation, Writing - original draft, Writing - review & editing, Visualization, Supervision.

Declaration of Competing Interest

None.

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