Evaluation of the Aptima™ HBV Quant Dx assay for semi-quantitative HBV viral load from dried blood spots

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Title page

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Abstract

Background: The detection and quantification of hepatitis B virus (HBV) DNA from dried blood spots (DBS) is a major tool for chronic hepatitis B management in resource-limited settings. This strategy fits in perfectly with the hepatitis control plan promoted by the World Health Organization. However, few commercial methods are validated for viral load (VL) measurement on DBS.

Objectives: Our objective was to evaluate the performance of the HBV VL measurement of the Aptima™ HBV Quant Dx assay on DBS compared to plasma samples on the Panther® platform (Hologic).

Study design: 266 whole blood samples for routine measurement were included. Five spots of 75 µL of whole blood were loaded onto a card before centrifugation and plasma settling.

Results: 149 samples were quantifiable and 117 were not detected. We achieved excellent linearity (r^2=0.994) over a wide range of measurements suitable for clinical practice, and a 95% lower limit of detection (LLOD-95%) at 2.65 $\log_{10}$ IU/mL (445 IU/mL). A good performance of this assay was observed for samples with HBV VL > LLOD-95% and 100% of samples were detected if HBV VL was above 2.95 $\log_{10}$ IU/mL. The correlation between the two matrices for quantitative VLs was good (r^2=0.978) with a very low bias (-0.002 $\log_{10}$ IU/mL).

Conclusion: The Aptima™ assay can properly detect and quantify HBV DNA in DBS, providing a satisfactory use in clinical monitoring and therapeutic decisions. DBS represents an excellent alternative to plasma, especially in resource-limited countries, while maintaining the performance and advantages of an automated technique.

Keywords: dried blood spots, hepatitis B virus, viral load, analysis performance, resource-limited settings

Background

Despite improvements in vaccines and targeted antiviral therapies, hepatitis B virus (HBV) remains a major public health concern. An estimated 300 million people worldwide live with HBV, and in 2013 47% of viral hepatitis-related mortalities resulted from acute or chronic hepatitis B cases associated with cirrhosis or hepatocellular carcinoma [1] with around 687,000 deaths. To eliminate HBV globally, the World Health Organization demonstrated the need for a global strategy combining extensive vaccination coverage of ≥90%, large-scale innovative prevention of mother-to-child transmission, and a "Test and Treat" strategy with access to antiviral treatments and high compliance [2].

To develop the "Test and Treat" strategy, the availability of diagnostic tools comprising serological tests (HBs Ag screening by rapid test) and also HBV viral load (VL) is essential whereas most patients live in resource-limited countries with restricted access to health centers. HBV VL measurement is crucial both to determine whether antiviral therapy should be recommended, and to assess the efficacy of antiviral treatment [3]. Although molecular biology is an expensive technique requiring adapted laboratories, the development of a new closed automated system could provide a solution for this analytical phase. But in resource-limited countries, the measurement of HBV VL and the management of the pre-analytical phase were only done at reference laboratories, if at all.

To obtain HBV VL and facilitate sample transport, the use of the dried blood spot (DBS) usually by capillary finger-stick is now largely recognized as a simple and easy alternative to venipuncture in local health centers without laboratories. However, for the purposes of this validation exercise, venous blood was spotted down. The DBS was widely shown to be stable for weeks at room temperature and down to -80°C [4–7]. The use of DBS for the collection and storage of blood samples and for VL quantification was validated in many studies [6–11]. However, manufacturers have not developed standardized protocols for using DBS with their

assays; thus the use of DBS is currently off-label and not approved by major regulatory authorities.

Objectives

Here, we developed the measurement of HBV VL in DBS on the automated Panther® (Hologic) using the Aptima™ HBV Quant Dx (i.e. Aptima HBV) compared to plasma samples.

Study design

Sample treatment

EDTA whole blood specimens were spotted onto a Whatman 903 protein saver card (GE Healthcare Life Sciences, Marlborough, MA) before centrifugation. Five spots of 75 µL were loaded [12], for a total of 5 DBS replicates per blood sample. DBS were air-dried for 24 hours, placed into zip-lock bags with desiccant packs and stored at -20°C, as recommended by the manufacturer, until analysis. After centrifugation for 10 min at 3,000 rpm, plasmas were stored at -20°C.

The Aptima™ HBV *Quant Dx assay*

The Aptima HBV was performed on a Panther® system (Hologic Inc., San Diego, CA, USA). The assay requires 700 µL and processes 500 µL of sample. The Aptima HBV amplifies the HBV S gene for genotypes A to H with a range of quantitation of 10 to 10^9 IU/mL.

Before DBS testing, each spot of 75 µL whole blood was manually cut extemporaneously from the DBS card with a pair of scissors and immediately placed with a metallic clip into a specimen aliquot tube (SAT, Hologic, Inc., San Diego, CA, USA) containing 1 mL of specimen transfer medium (STM, Hologic, Inc., San Diego, CA, USA). Between each DBS cut, the pair of scissors and metallic clip were disinfected in Hexanios® solution [13]. Specimen tubes were then capped and incubated at room temperature for 30 min in a rocker platform. They were then briefly centrifuged (2 min at 3,000 rpm) before HBV VL measurement with the Aptima HBV assay (research use only).

To consider the multiple dilution factors and to correct the volumetric differences between whole blood and plasma, we needed to apply a conversion factor of 22.99 to all HBV VL results in IU/mL and $\log_{10}$ IU/mL reported by Panther® for the DBS testing. The volume adjustment calculation was previously defined by Sahoo et al [14] and calculated as follows: volume of STM (1,000 µL) / [(volume of whole blood collected in DBS in µL) x (1-estimated

hematocrit)] i.e. 1000 / (75 x 0.58). A value of 42% for the hematocrit was used as it is the mean adult value determined by the National Health and Nutrition Examination Survey [15]. For detectable but unquantifiable HBV DNA below the lower limit of quantitation (LLOQ) (detected <10 IU/mL), as described and done by others and to allow statistical analysis [7], we took into consideration a midpoint value (5 IU/mL) between zero and the lower limit of quantitation (10 IU/mL). Results where all five DBS replicates were unquantifiable or undetected were assigned a value of "target not detected". Where all five replicates gave quantifiable results, the average of the 5 replicates was used. Where at least one DBS out of five replicates gave a quantifiable result, we assigned a value "< lower limit of detection (LLOD)" [11].

Linearity

Linearity was determined using a fresh HBV-negative EDTA whole blood sample that was spiked with an HBV-positive sample (HBV VL at 9.19 $\log_{10}$ IU/mL with Aptima HBV). Serial dilutions were performed to obtain eight range points of linearity ranging from 2 to 9 $\log_{10}$ IU/mL. Each range point was loaded onto a card in five replicates. The assays were then carried out during the same run.

Lower limit of detection (LLOD) and precision analysis for DBS samples

The 95% LLOD on DBS samples was determined using the same spiked sample as previously described for linearity (same HBV VL). Five range points between 2.1 and 3.7 $\log_{10}$ IU/mL were loaded 30 times onto the card. The tests were then performed in three different runs (ten replicates per run). Repeatability was performed at two levels using two HBV-positive samples: one sample with high VL of 7.68 $\log_{10}$ IU/mL and one sample with low VL of 3.43 $\log_{10}$ IU/mL. Each sample was loaded onto the card in 15 replicates.

Clinical specimens

266 EDTA whole blood samples for HBV VL routine measurement were consecutively collected in the laboratory of virology and the study was approved by the Institutional Board of the University Hospital of Angers.

HBV genotype determination

In a subset of samples, HBV genotype was available and determined using the method described by Villeneuve et al. [16].

Statistical analysis

All statistical analyses were performed using IBM® SPSS® 15.0 Statistics (Statistical Package for Social Sciences, IBM Corp., Chicago, IL). The 95% LLOD was calculated using probit analysis. Precision was analyzed in Microsoft Excel 2010 using the formula described by Chesher [17]. A linear bivariate correlation was performed between plasma and DBS (Spearman's correlation coefficient). Additionally, a Bland Altman analysis was done to assess bias and agreement between plasma and DBS.

Results

To assess the measurement of HBV VL on DBS using the Aptima HBV assay, we first did a performance evaluation by testing serial dilution of a fresh HBV negative plasma spiked with an HBV-positive sample (n=8 range points) covering the range of quantification given by the manufacturer for plasma samples. We obtained five results for each range point excepted for two: 9 $\log_{10}$ IU/mL (n=4 and one invalid result) and 2 $\log_{10}$ IU/mL (n=4 and one "not detected" result). The linearity study shows a correlation coefficient r^2 at 0.994 associated with low coefficient of variation (CV%) for all range points (Figure 1).

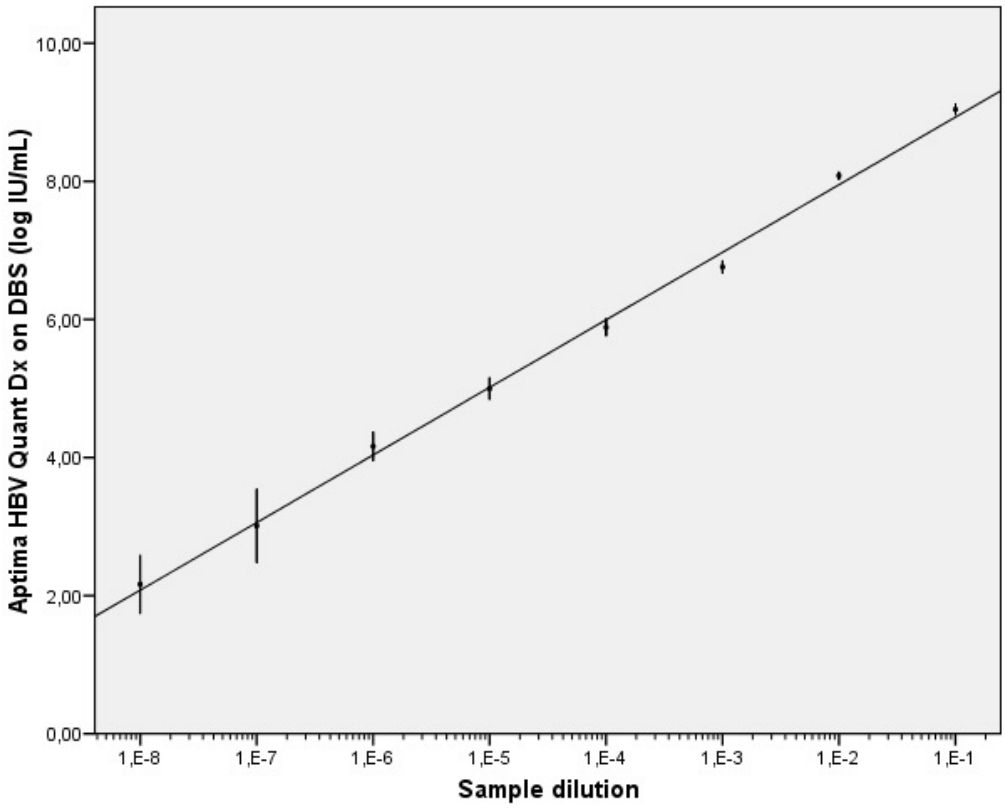


Figure 1: Linearity study. Results of the 8 range points of linearity ranging from 2 to 9 $\log_{10}$ IU/mL tested in 5 replicates in one run. The coefficients of variation varied from 0.32 to 9.70%. The error bar corresponded to 1.96*standard deviation. Linear equation was $y=0.425*\log_{10}(x) + 0.909$ and $r^2=0.994$ ($p<0.005$).

In a second step, we intended to assess the assay performance by determining the LLOD threshold for whole blood on DBS. We used the same spiked sample as previously described for linearity. The LLOD-95% was estimated at 2.65 log_{10} IU/mL or 445 IU/mL (95% Confidence Interval [CI]: 2.58 - 2.74 log_{10} IU/mL or 384 - 553 IU/mL). The within-run, between-day, and within-laboratory (total) imprecision levels (i.e. standard deviations) were 0.17 log_{10}, 0.32 log_{10}, and 0.17 log_{10} IU/mL, respectively, for DBS containing a nominal HBV concentration of 3.01 log_{10} IU/mL, the lowest concentration at which all replicates were within the quantifiable range of the assay. The CV% of low level obtained for repeatability using 15 replicates was 4.12% (3.40 ± 0.14 log_{10} IU/mL). The CV% of high level was 1.21% (7.42 ± 0.09 log_{10} IU/mL). There were no outliers.

Finally, we compared assay performance between plasma and DBS. 266 samples were included consecutively between January and April 2019. A quantifiable HBV VL was found for 149 plasma samples (56%) from 1.00 to 8.91 log_{10} IU/mL (average=7.59 log_{10} IU/mL), 33 plasma samples had a detected HBV VL <10 IU/mL (12%) and 84 plasma samples had a "not detected" HBV VL (32%). HBV DNA was quantified in 86 DBS out of 149 quantified plasma (58%), with plasma VL ranging from 2.70 to 8.73 log_{10} IU/mL (average=7.65 log_{10} IU/mL). 26 samples that were detected/not quantified in DBS (under the DBS LLOD threshold) were quantified using the plasma sample and ranged from 2.10 to 2.95 log_{10} IU/mL (with only 7/26 samples >2.65 log_{10} IU/mL). Then, 37 samples that were quantified in plasma were not detected in DBS. Among these samples, HBV DNA was detected and quantified in plasma with VL ranging from 1.00 to 2.58 log_{10} IU/mL (average=1.92 ± 0.27 log_{10} IU/mL) and below the DBS LLOD threshold (Table 1).

		DBS (Aptima HBV)			Total
		Not detected	<LLOD[1]	≥LLOD	
Plasma sample (Aptima HBV)	Not detected	81	3	0	84
	< 10 IU/mL	30	3	0	33
	≥ 10 IU/mL	37	26	86	149
Total		148	32	86	266

LLOD[1] corresponds to the 95% lower limit of detection

Table 1: Detection and quantification of HBV DNA in DBS versus plasma sample.

The agreement of detection of HBV DNA between plasma and DBS was at 100.0% (95% CI: 95.3-100%) for HBV VL ≥2.95 $\log_{10}$ IU/mL. The DBS detection rate was 8.6% (95% CI: 4.6-15.5%) for quantifiable HBV VL <2.95 $\log_{10}$ IU/mL.

For the 86 plasma samples with quantifiable DNA in plasma/DBS (average [DBS]=7.83 $\log_{10}$ IU/mL; 2.50 - 8.91 $\log_{10}$ IU/mL), performance of the Aptima HBV in the paired DBS gave similar results that showed a strong and positive correlation (r^2=0.978) which was significant ($p<0.001$) (Figure 2). There were no outliers for HBV VL. The Bland Altman plot illustrates the agreement between plasma and DBS viral load results (Figure 3). We showed a bias of -0.002 $\log_{10}$ IU/mL between plasma and HBV DNA concentrations in DBS. 95.3% of values were included in the limits of agreement and indicated that the difference between plasma and DBS was between -0.435 and 0.431 $\log_{10}$ IU/mL. There were no outliers outside the limits of agreement. Bland-Altman analysis showed that HBV DNA tended to be higher in plasma than DBS for samples with HBV DNA above 7 $\log_{10}$ IU/mL (bias of 0.241 $\log_{10}$ IU/mL, n=14 vs -0.114 $\log_{10}$ IU/mL, n=72).

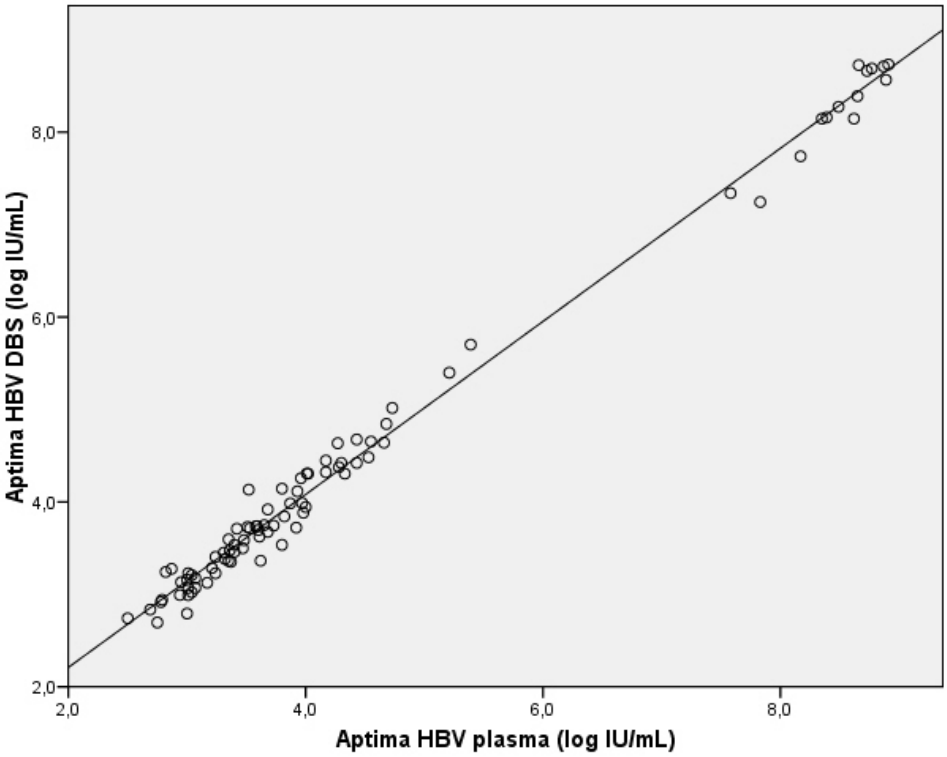


Figure 2: Correlation analysis between DBS and plasma sample with the Aptima HBV assay (n=86), y=0.936x + 0.338. r^2=0.978; the diagonal line represents identical results.

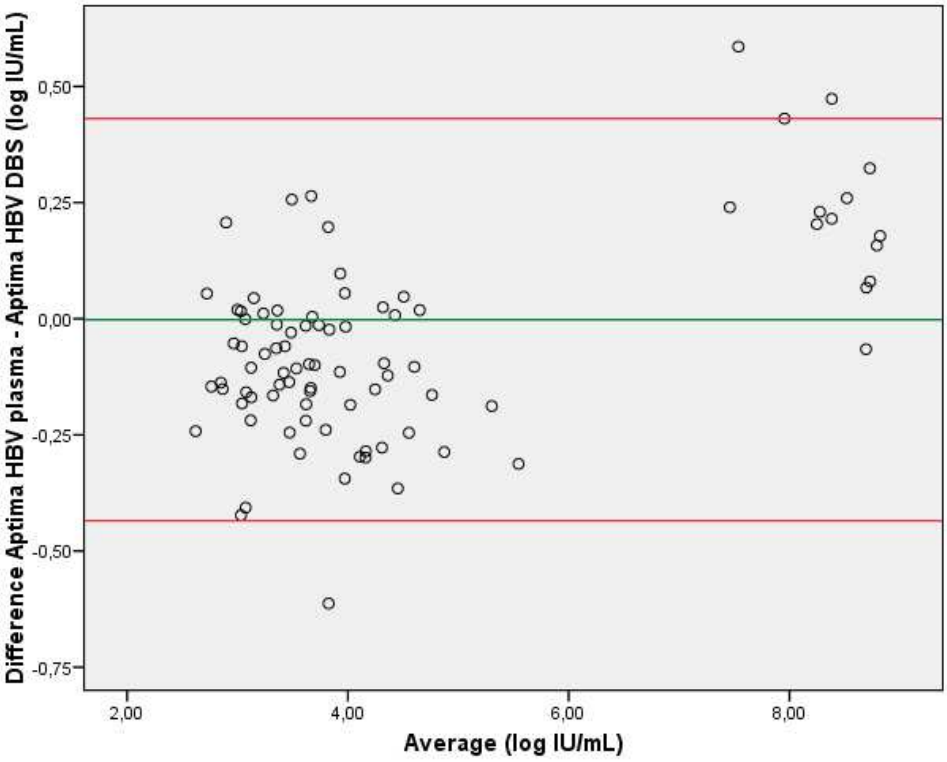
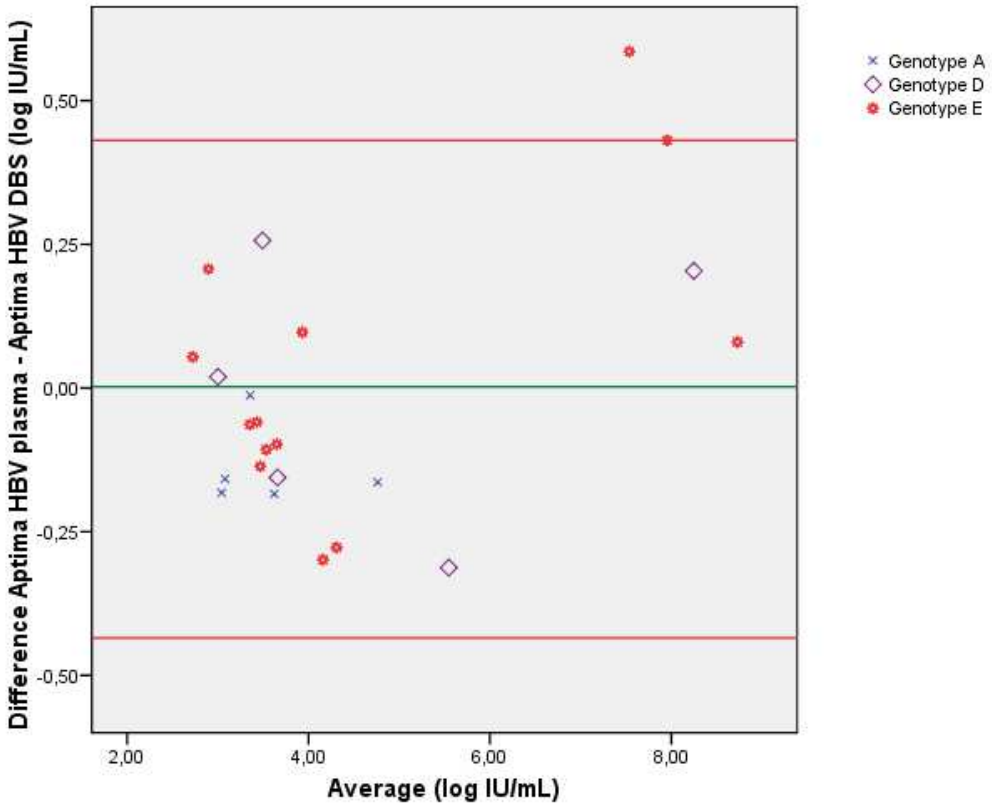


Figure 3: Bland-Altman analysis to show bias and agreement for HBV DNA quantification in plasma samples compared with DBS samples (n=86 plasma/DBS pairs with HBV quantifiable VL) on the Aptima HBV assay. The green line represents bias (-0.002 log_{10} IU/mL); the two red lines represent the 95% limit of agreement (-0.435 to 0.431 log_{10} IU/mL) (mean of differences ± 1.96 x standard deviation of differences).

HBV genotype was available for 59 samples among 266, as follows: HBV-A (n=14), HBV-B (n=1), HBV-C (n=4), HBV-D (n=11), HBV-E (n=29). Bland-Altman analysis was showed for the three most prevalent genotypes in Figure 4.



194

195 **Figure 4**: Bland-Altman analysis to show bias and agreement for samples with HBV
196 genotype available in plasma samples compared with DBS samples (n=23 quantifiable
197 plasma/DBS pairs) on the Aptima™ assay. Bland-Altman plot analysis showed a low bias of -
198 0.037 $\log_{10}$ IU/mL (n=13, 95% limit of agreement: -0.40 – 0.55 $\log_{10}$ IU/mL), -0.003 $\log_{10}$
199 IU/mL (n=5, 95% limit of agreement: -0.47 – 0.47 $\log_{10}$ IU/mL) and 0.073 $\log_{10}$ IU/mL (n=5,
200 95% limit of agreement: -0.40 – 0.55 $\log_{10}$ IU/mL) for HBV genotypes E, D and A
201 respectively. The green line represents bias (-0.002 $\log_{10}$ IU/mL); the two red lines represent
202 the 95% limit of agreement -0.435 to 0.431 $\log_{10}$ IU/mL.

Discussion

Access to screening and monitoring of chronic HBV infection in low-resource settings currently remains very limited; meanwhile, these countries remain endemic areas for this infection. Use of DBS as an alternative sample collection technique can increase testing uptake where venipuncture is a barrier to testing. In this study, we evaluated the performance of the Aptima HBV assay for HBV DNA detection and quantification from EDTA whole blood samples loaded in DBS compared to plasma. To our knowledge, this is the first study to compare the Aptima HBV in DBS and plasma samples. Previous studies have shown the utility of Aptima™ HIV and HCV Quant Dx Assay on the Panther® system for quantification of HIV-1 and HCV from DBS [7,11,14,18]. Furthermore, previous studies described the comparison of HBV DNA detection in DBS vs. plasma with other nucleic acid amplification testing methods [6,19,20].

We observed an excellent linearity over a wide range of measurements suitable for clinical practice, and a detection threshold (LLOD-95%) of 445 IU/mL with an important sample size. Our LLOD-95% was lower than the range defined by Lange's meta-analysis (900-4,000 IU/mL) [20], but similar to the LLOD described by Stene-Johansen et al using the Abbott RealTime HBV assay with only 26 samples [6]. The LLOD-95% was higher than when analyzing plasma and met our expectations due to a much lower sample volume spotted on DBS (i.e. 75 µL vs 500 µL with plasma). Others studies reported this issue [19,21], but so far, none of these studies mentioned a suitable alternative to increase the sensitivity of DBS and obtain the same LLOD threshold as when using plasma. Using this LLOD threshold, detection sensitivity was impacted because 37 quantified plasma samples were not detected in DBS. These results correspond to very low VL and were $\leq$2.58 $\log_{10}$ IU/mL, i.e. below the DBS LLOD threshold.

228 Moreover, using a cut-off of 2.95 log_{10} IU/mL, 100% of DBS were correctly assigned. So, our
229 LLOD threshold is well-adapted to the current therapeutic decision threshold of 2,000 IU/mL
230 or 3.30 log_{10} IU/mL (therapeutic decision cut-off from AASLD guidelines) and for viral
231 resistance testing (current assays require HBV DNA level >1,000 IU/mL or 3.00 log_{10} IU/mL)
232 [3] and DBS might be useful to assess treatment eligibility in CHB. DBS is ill-suited in
233 settings where a low VL may be clinically relevant, such as blood transfusion screening,
234 molecular qualification of organ donors or to detect occult infections [6].
235 A strong correlation (Spearman correlation coefficient=0.978, $p<0.01$) was observed between
236 plasma, and higher than the coefficient of 0.84 reported in an equivalent study using the
237 COBAS Taqman/COBAS AmpliPrep method [22]. As the bias was very low (-0.002 log_{10}
238 IU/mL) and below the maximum variation currently accepted (0.50 log_{10} IU/mL) [23,24],
239 DBS probably have adequate sensitivity to detect virological breakthrough of clinical
240 relevance (defined as a 1.00 log_{10} IU/mL increase from nadir). Furthermore, although manual
241 preparation of DBS is labour intensive compared to loading tubes in a machine,
242 contamination is unlikely according to our results.
243
244 To estimate the impact of the genetic diversity for this assay, 59 HBV genotypes were
245 described out of 266 samples included. This information was unfrequently evaluated in other
246 studies [19,25]. The main genotypes observed were genotypes A, D and E and this
247 distribution was similar to what was observed in France [19,26,27]. Taking the HBV
248 genotype into account, the difference observed between plasma and HBV VL in DBS was
249 equivalent to the values observed on all HBV quantified samples. The genotype does not
250 seem to affect VL measurement in DBS because the bias was very low for genotypes A, D
251 and E. This result was consistent with a previous study performed with plasma samples

252 representing all HBV genotypes [28]. However, a large batch of samples with HBV
253 genotypes available would be useful to confirm these results, notably for genotypes B and C.
254
255 Our study has several limitations. Firstly, we used EDTA whole blood samples onto filter
256 paper rather than patient collected finger-sticks, as usually done to evaluate performance of
257 nucleic acid test [7,11]. Analytical sensitivity is reduced in DBS compared to fresh plasma
258 (notably for low HBV VL) due to small input volumes [29]. It may be possible to increase
259 sensitivity by adding multiple dried blood spots and by using an adapted volume of elution
260 buffer (not evaluated here). Our study did not assess storage conditions of DBS considering
261 that it has been largely assessed in other studies [6,30,31].
262
263 In conclusion, our data suggests that the Aptima HBV test detects and quantify HBV DNA
264 from a DBS with good performance for samples with HBV VL >LLOD and for 100% of
265 samples with HBV VL >2.95 $\log_{10}$ IU/mL. Importantly, this work was a complete study with
266 five replicates per whole blood sample allowing the assessment of intra- and inter-assay
267 variability. The main strength of our study was the important sample size (n=266) covering a
268 large range of "real-world" VL levels with rather representative genetic diversity. DBS are an
269 excellent alternative to plasma samples, especially in resource-limited countries.
270

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