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1 **Title page**

2 **Title:** Evaluation of the Aptima™ HBV Quant Dx Assay for semi-quantitative HBV viral load
3 from dried blood spots

4

5 **Author names and affiliations:**

6 Steven Roger^a, Caroline Lefeuvre^{a,b}, Marine Grison^a, Alexandra Ducancelle^{a,b}, Françoise
7 Lunel-Fabiani^{a,b}, Adeline Pivert^{a,b}, Hélène Le Guillou-Guillemette^{a,b}

8 ^a*Virology Department, Angers University Hospital, Angers, France*

9 ^b*HIFIH Laboratory EA 3859, LUNAM, Angers, France*

10

11 **Corresponding author:** Hélène Le Guillou-Guillemette

12 PharmD, PhD

13 E-mail address: HeLeguillou@chu-angers.fr

14 Postal address: Virology Department, Angers University Hospital, HIFIH Laboratory EA
15 3859, LUNAM, 4, rue Larrey 49000 Angers, France

16 Telephone number: (+33) 02 41 35 47 09

17

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20 **Declaration of interest:**

21 Hologic provided Aptima™ Quant Dx Viral Load assays and STM, and the authors thank
22 Tom Oosterlaken from Hologic for funding assistance. The sponsor had no role in data
23 collection, analysis and interpretation of the results, decision to publish or preparation of the
24 manuscript.

25 HLGG has received lecture fees from Hologic.

1 **Abstract**

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

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

10 *Study design:* 266 whole blood samples for routine measurement were included. Five spots of
11 75 µL of whole blood were loaded onto a card before centrifugation and plasma settling.

12 *Results:* 149 samples were quantifiable and 117 were not detected. We achieved excellent
13 linearity ($r^2=0.994$) over a wide range of measurements suitable for clinical practice, and a
14 95% lower limit of detection (LLOD-95%) at 2.65 \log_{10} IU/mL (445 IU/mL). A good
15 performance of this assay was observed for samples with HBV VL > LLOD-95% and 100%
16 of samples were detected if HBV VL was above 2.95 \log_{10} IU/mL. The correlation between
17 the two matrices for quantitative VLs was good ($r^2=0.978$) with a very low bias (-0.002 \log_{10}
18 IU/mL).

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

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

25

26 **Background**

27 Despite improvements in vaccines and targeted antiviral therapies, hepatitis B virus (HBV)
28 remains a major public health concern. An estimated 300 million people worldwide live with
29 HBV, and in 2013 47% of viral hepatitis-related mortalities resulted from acute or chronic
30 hepatitis B cases associated with cirrhosis or hepatocellular carcinoma [1] with around
31 687,000 deaths. To eliminate HBV globally, the World Health Organization demonstrated the
32 need for a global strategy combining extensive vaccination coverage of $\geq 90\%$, large-scale
33 innovative prevention of mother-to-child transmission, and a “Test and Treat” strategy with
34 access to antiviral treatments and high compliance [2].

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

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

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

53

54 **Objectives**

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

58

59 **Study design**

60 *Sample treatment*

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

67 *The Aptima™ HBV Quant Dx assay*

68 The Aptima HBV was performed on a Panther® system (Hologic Inc., San Diego, CA, USA).
69 The assay requires 700 μ L and processes 500 μ L of sample. The Aptima HBV amplifies the
70 HBV S gene for genotypes A to H with a range of quantitation of 10 to 10⁹ IU/mL.

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

79 To consider the multiple dilution factors and to correct the volumetric differences between
80 whole blood and plasma, we needed to apply a conversion factor of 22.99 to all HBV VL
81 results in IU/mL and log₁₀ IU/mL reported by Panther® for the DBS testing. The volume
82 adjustment calculation was previously defined by Sahoo et al [14] and calculated as follows:
83 volume of STM (1,000 μ L) / [(volume of whole blood collected in DBS in μ L) x (1-estimated

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

94 ***Linearity***

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

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

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

107

108 ***Clinical specimens***

109 266 EDTA whole blood samples for HBV VL routine measurement were consecutively
110 collected in the laboratory of virology and the study was approved by the Institutional Board
111 of the University Hospital of Angers.

112 *HBV genotype determination*

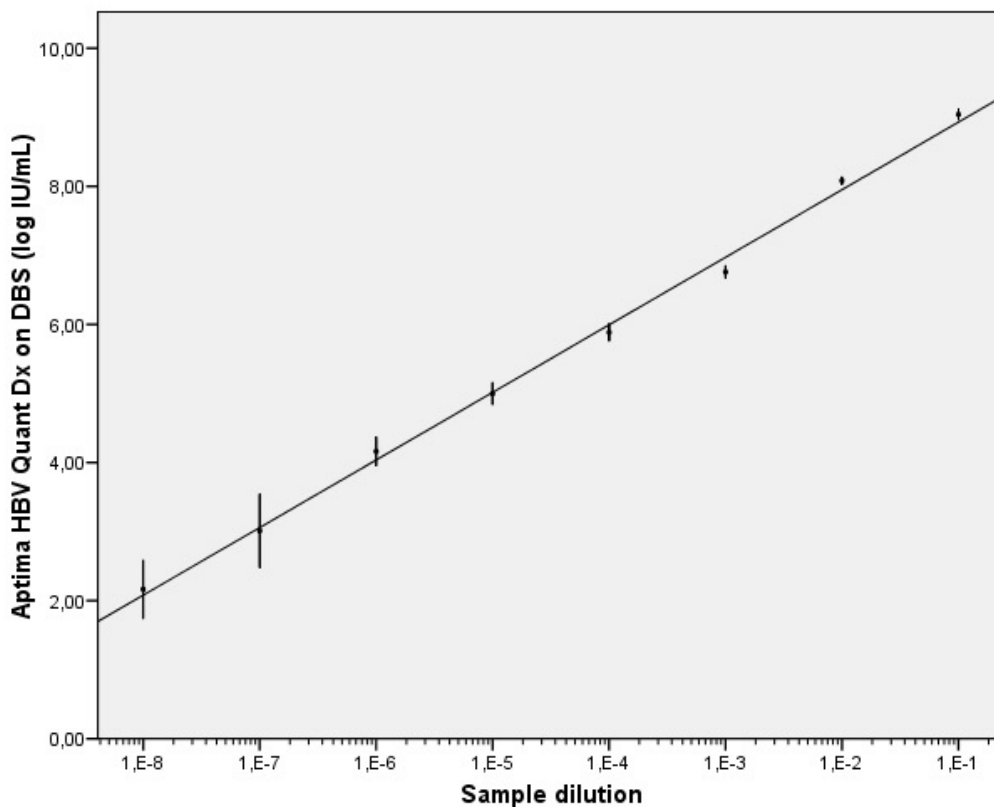
113 In a subset of samples, HBV genotype was available and determined using the method
114 described by Villeneuve et al. [16].

115 *Statistical analysis*

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

122 **Results**

123 To assess the measurement of HBV VL on DBS using the Aptima HBV assay, we first did a
124 performance evaluation by testing serial dilution of a fresh HBV negative plasma spiked with
125 an HBV-positive sample (n=8 range points) covering the range of quantification given by the
126 manufacturer for plasma samples. We obtained five results for each range point excepted for
127 two: 9 log₁₀ IU/mL (n=4 and one invalid result) and 2 log₁₀ IU/mL (n=4 and one “not
128 detected” result). The linearity study shows a correlation coefficient r² at 0.994 associated
129 with low coefficient of variation (CV%) for all range points (Figure 1).



130

131 **Figure 1:** Linearity study. Results of the 8 range points of linearity ranging from 2 to 9 log₁₀
132 IU/mL tested in 5 replicates in one run. The coefficients of variation varied from 0.32 to
133 9.70%. The error bar corresponded to 1.96*standard deviation. Linear equation was
134 $y=0.425*\log_{10}(x) + 0.909$ and $r^2=0.994$ ($p<0.005$).

135

136 In a second step, we intended to assess the assay performance by determining the LLOD
137 threshold for whole blood on DBS. We used the same spiked sample as previously described
138 for linearity. The LLOD-95% was estimated at 2.65 log₁₀ IU/mL or 445 IU/mL (95%
139 Confidence Interval [CI]: 2.58 - 2.74 log₁₀ IU/mL or 384 - 553 IU/mL). The within-run,
140 between-day, and within-laboratory (total) imprecision levels (i.e. standard deviations) were
141 0.17 log₁₀, 0.32 log₁₀, and 0.17 log₁₀ IU/mL, respectively, for DBS containing a nominal HBV
142 concentration of 3.01 log₁₀ IU/mL, the lowest concentration at which all replicates were
143 within the quantifiable range of the assay. The CV% of low level obtained for repeatability
144 using 15 replicates was 4.12% (3.40 ± 0.14 log₁₀ IU/mL). The CV% of high level was 1.21%
145 (7.42 ± 0.09 log₁₀ IU/mL). There were no outliers.

146

147 Finally, we compared assay performance between plasma and DBS. 266 samples were
148 included consecutively between January and April 2019. A quantifiable HBV VL was found
149 for 149 plasma samples (56%) from 1.00 to 8.91 log₁₀ IU/mL (average=7.59 log₁₀ IU/mL), 33
150 plasma samples had a detected HBV VL <10 IU/mL (12%) and 84 plasma samples had a “not
151 detected” HBV VL (32%). HBV DNA was quantified in 86 DBS out of 149 quantified
152 plasma (58%), with plasma VL ranging from 2.70 to 8.73 log₁₀ IU/mL (average=7.65 log₁₀
153 IU/mL). 26 samples that were detected/not quantified in DBS (under the DBS LLOD
154 threshold) were quantified using the plasma sample and ranged from 2.10 to 2.95 log₁₀ IU/mL
155 (with only 7/26 samples >2.65 log₁₀ IU/mL). Then, 37 samples that were quantified in plasma
156 were not detected in DBS. Among these samples, HBV DNA was detected and quantified in
157 plasma with VL ranging from 1.00 to 2.58 log₁₀ IU/mL (average=1.92 ± 0.27 log₁₀ IU/mL)
158 and below the DBS LLOD threshold (Table 1).

159

160

161

| | | 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 |

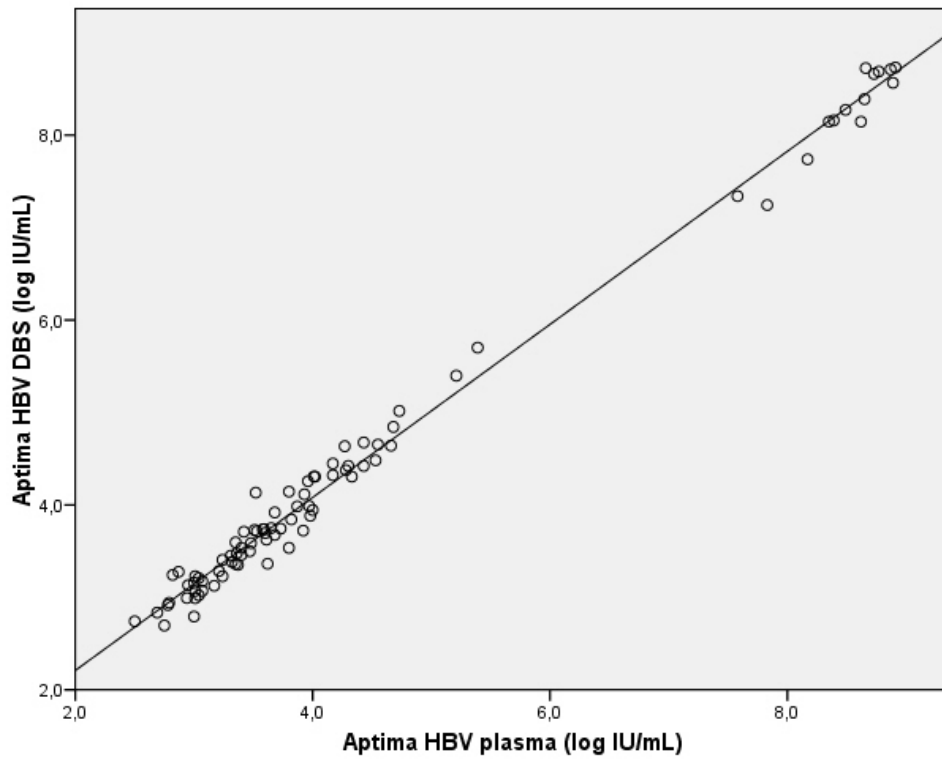
162 LLOD¹ corresponds to the 95% lower limit of detection

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

164

165 The agreement of detection of HBV DNA between plasma and DBS was at 100.0% (95% CI:
166 95.3-100%) for HBV VL $\geq 2.95 \log_{10}$ IU/mL. The DBS detection rate was 8.6% (95% CI: 4.6-
167 15.5%) for quantifiable HBV VL $< 2.95 \log_{10}$ IU/mL.

168 For the 86 plasma samples with quantifiable DNA in plasma/DBS (average [DBS]=7.83 \log_{10}
169 IU/mL; 2.50 - 8.91 \log_{10} IU/mL), performance of the Aptima HBV in the paired DBS gave
170 similar results that showed a strong and positive correlation ($r^2=0.978$) which was significant
171 ($p<0.001$) (Figure 2). There were no outliers for HBV VL. The Bland Altman plot illustrates
172 the agreement between plasma and DBS viral load results (Figure 3). We showed a bias of -
173 0.002 \log_{10} IU/mL between plasma and HBV DNA concentrations in DBS. 95.3% of values
174 were included in the limits of agreement and indicated that the difference between plasma and
175 DBS was between -0.435 and 0.431 \log_{10} IU/mL. There were no outliers outside the limits of
176 agreement. Bland-Altman analysis showed that HBV DNA tended to be higher in plasma than
177 DBS for samples with HBV DNA above 7 \log_{10} IU/mL (bias of 0.241 \log_{10} IU/mL, n=14 vs -
178 0.114 \log_{10} IU/mL, n=72).



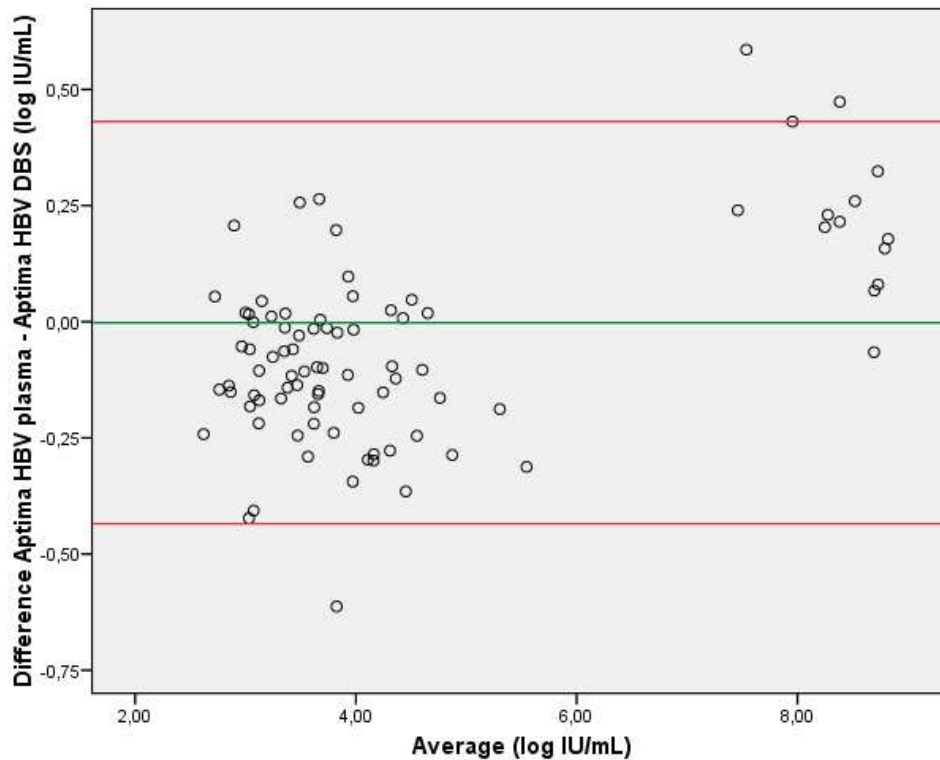
179

180 **Figure 2:** Correlation analysis between DBS and plasma sample with the Aptima HBV assay

181 (n=86), $y=0.936x + 0.338$. $r^2=0.978$; the diagonal line represents identical results.

182

183

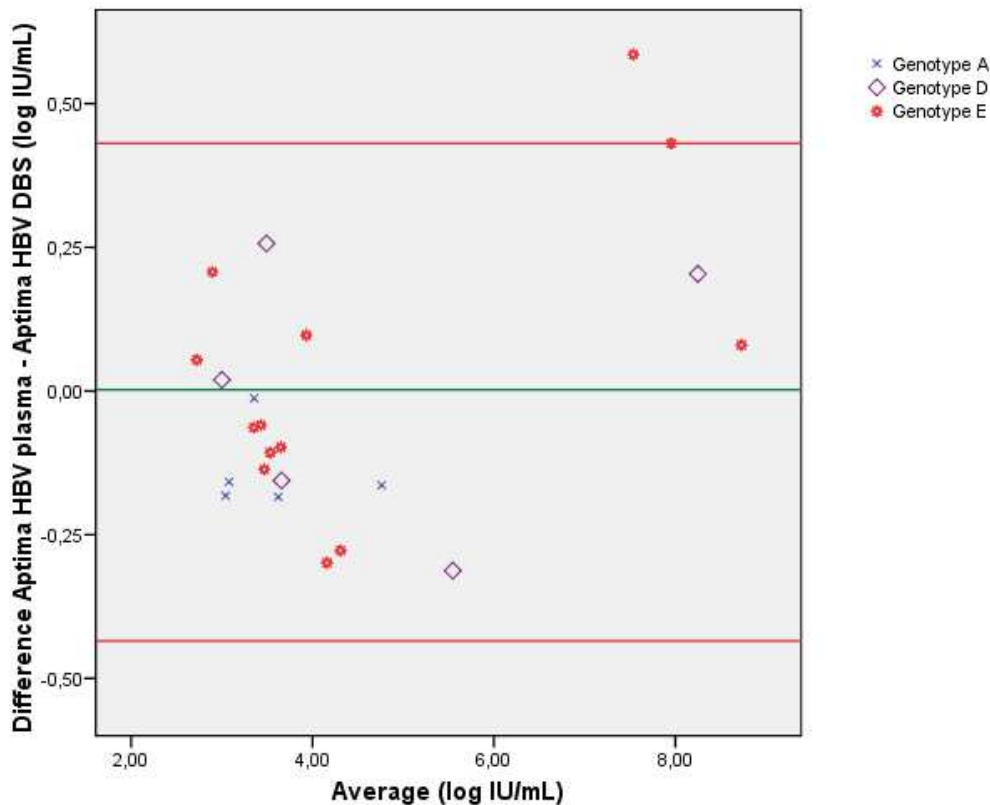


184

185 **Figure 3:** Bland-Altman analysis to show bias and agreement for HBV DNA quantification in
 186 plasma samples compared with DBS samples (n=86 plasma/DBS pairs with HBV quantifiable
 187 VL) on the Aptima HBV assay. The green line represents bias (-0.002 log₁₀ IU/mL); the two
 188 red lines represent the 95% limit of agreement (-0.435 to 0.431 log₁₀ IU/mL) (mean of
 189 differences ± 1.96 x standard deviation of differences).

190

191 HBV genotype was available for 59 samples among 266, as follows: HBV-A (n=14), HBV-B
 192 (n=1), HBV-C (n=4), HBV-D (n=11), HBV-E (n=29). Bland-Altman analysis was showed
 193 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 log₁₀ IU/mL (n=13, 95% limit of agreement: -0.40 – 0.55 log₁₀ IU/mL), -0.003 log₁₀
 199 IU/mL (n=5, 95% limit of agreement: -0.47 – 0.47 log₁₀ IU/mL) and 0.073 log₁₀ IU/mL (n=5,
 200 95% limit of agreement: -0.40 – 0.55 log₁₀ IU/mL) for HBV genotypes E, D and A
 201 respectively. The green line represents bias (-0.002 log₁₀ IU/mL); the two red lines represent
 202 the 95% limit of agreement -0.435 to 0.431 log₁₀ IU/mL.

203 **Discussion**

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

215
216 We observed an excellent linearity over a wide range of measurements suitable for clinical
217 practice, and a detection threshold (LLOD-95%) of 445 IU/mL with an important sample size.
218 Our LLOD-95% was lower than the range defined by Lange's meta-analysis (900-4,000
219 IU/mL) [20], but similar to the LLOD described by Stene-Johansen et al using the Abbott
220 RealTime HBV assay with only 26 samples [6]. The LLOD-95% was higher than when
221 analyzing plasma and met our expectations due to a much lower sample volume spotted on
222 DBS (i.e. 75 µL vs 500 µL with plasma). Others studies reported this issue [19,21], but so far,
223 none of these studies mentioned a suitable alternative to increase the sensitivity of DBS and
224 obtain the same LLOD threshold as when using plasma. Using this LLOD threshold,
225 detection sensitivity was impacted because 37 quantified plasma samples were not detected in
226 DBS. These results correspond to very low VL and were $\leq 2.58 \log_{10}$ IU/mL, i.e. below the
227 DBS LLOD threshold.

228 Moreover, using a cut-off of 2.95 log₁₀ 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 log₁₀ 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 log₁₀ 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 log₁₀
238 IU/mL) and below the maximum variation currently accepted (0.50 log₁₀ IU/mL) [23,24],
239 DBS probably have adequate sensitivity to detect virological breakthrough of clinical
240 relevance (defined as a 1.00 log₁₀ 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 log₁₀ 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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