

## ORIGINAL ARTICLE

# Comparison of four extraction methods for the detection of hepatitis B virus DNA in dried blood spot samples

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## Abstract

The dried blood spot (DBS) samples are a useful resource for viral DNA isolation and important in increasing access to HBV diagnosis. However, the choice of the DNA extraction method is crucial for reliable results. We compared the reliability of four DNA extraction methods using DBS samples for the qualitative and quantitative detection of HBV. A panel of serially diluted HBV DNA in whole blood was spotted onto filter paper (Whatman 903 paper and Whatman FTA cards). Four methods were used to extract DNA: QIAamp<sup>®</sup> DNA Blood Mini Kit (Qiagen); High Pure Viral Nucleic Acid Kit (Roche); Invisorb Spin Blood Midi Kit (Invitex), and DBS Genomic DNA Isolation Kit (Norgen Biotek). Two qualitative PCRs for the core and surface gene regions of HBV were used, and in-house real-time PCR was also evaluated. It was possible to detect HBV DNA using all extraction and PCR protocols. The lowest limit of detection was found using Whatman 903 paper, Roche extraction, and qualitative PCR (20 copies of HBV DNA per ml) for the surface/polymerase HBV region. In the case of in-house real-time PCR, the lowest limit of detection was found using both Roche and Qiagen assays (estimated  $2 \times 10^3$  copies per ml). These results suggest the importance of both the extraction method and PCR protocol in detecting HBV DNA in DBS. This study provides insights into the utility of DBS samples in HBV molecular diagnosis and their feasibility in low resource areas where cold storage and transportation may be difficult.

## KEYWORDS

DBS, DNA extraction, hepatitis B, PCR, real-time PCR

## 1 | INTRODUCTION

Hepatitis B is a substantial, worldwide health problem. The World Health Organization (WHO) estimates that around two billion people have been exposed to hepatitis B virus (HBV), while 257 million people are chronically infected (WHO, 2017). Thus, since 2015, the WHO has focused on the importance of controlling HBV

transmission and disease progression, especially in regions where hepatitis B is endemic (WHO, 2015, 2016).

Viral diagnosis is made by detecting antibodies against the virus and HBV antigens. However, the treatment of infected individuals relies on the determination of viral load (Silva et al., 2011; Villar et al., 2015; WHO, 2015, 2016, 2017). According to EASL guidelines, all patients with HBAg-positive or HBAg-negative chronic hepatitis B,

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defined by HBV DNA (2000 IU/ml), ALT > ULN, and at least moderate liver necroinflammation or fibrosis, should be treated (EASL, 2017). Access to HBV molecular diagnosis could be difficult for people living in remote areas with a high prevalence of HBV. As an alternative, the dried blood spot sample (DBS) has been recommended for field studies, because it does not need special storage conditions or technical expertise for its collection. Also, the sample does not require immediate processing, as it must be dried at room temperature and packed in silica-containing plastics. Thus, it can be classified and transported as a lower biological risk than the transport of, for example, serum samples (McDade, 2014; Smit et al., 2014). Moreover, previous studies have suggested that due to its good sensitivity and specificity compared to the gold standard diagnostic samples (Mohamed et al., 2013; Smit et al., 2014; Vinikoor et al., 2015), DBS samples represent a viable alternative in HBV diagnosis (Boa-Sorte et al., 2014; Mayer et al., 2012; Mössner et al., 2016; Villar et al., 2011). However, little is known about the performance of extraction protocols for HBV DNA from DBS. The DNA extraction method must be chosen carefully, as small quantities of nucleic acids can cause false-negative results. Moreover, the extraction method must be able to remove substances that could impair molecular analysis (Sjöholm et al., 2007; Smit et al., 2014).

This study determines the best HBV DNA extraction method using DBS samples along with qualitative and quantitative methods to assess HBV diagnostic feasibility in low resource areas.

## 2 | MATERIALS AND METHODS

### 2.1 | Panel of biological samples for evaluating different DNA extraction methods

A sample comprised whole blood from healthy individuals without serological markers for HBV or hepatitis C virus (HCV) infection (negative for HBsAg, anti-HBc, and anti-HCV) was divided to produce a negative control sample and a test sample spiked with HBV DNA positive serum (viral load of  $2 \times 10^8$  copies/ml). The viral load of HBV DNA positive serum was determined using *Abbott RealTime HBV* (Abbott Diagnostics), and this determination was performed in our laboratory. A positive HBV serum sample was used to create a 10-fold serial dilution in whole blood, giving HBV concentrations ranging from  $2 \times 10^7$  to  $2 \times 10^0$  copies/ml, after conversion.

Negative and positive samples were distributed on two types of filter paper: 125  $\mu$ l of blood was spotted in each circle of Whatman FTA cards (GE Healthcare, Life Sciences), and 75  $\mu$ l of blood was spotted in each circle of Whatman 903 Paper (GE Healthcare, Life Sciences) according to the manufacturer's instructions. Four circles of Whatman FTA Cards and five circles of Whatman 903 Paper were prepared. After blood spotting, the DBS samples were dried at room temperature for four hours and placed into zip-locked bags with silica gel desiccant sachets and stored at  $-20^\circ\text{C}$  until testing (less than 14 days of storage). HBV-negative DBS samples were tested to monitor cross-contamination in the assay, and the serial HBV dilution panel was tested to evaluate the analytical sensitivity.

To evaluate the methods in volunteers, the DBS and serum samples from 10 individuals (five HBV-infected and five uninfected) were tested using an optimized protocol. All samples included in the study came from the biorepository of the Viral Hepatitis Laboratory of FIOCRUZ.

### 2.2 | Viral DNA extraction methods

Each DBS sample from the serial dilution panel, distributed on Whatman FTA Cards and Whatman 903 paper, was submitted to four DNA extraction kits according to the manufacturer's instructions, but with some adaptations.

We evaluated commercial assays: QIAamp<sup>®</sup> DNA Blood Mini Kit (Qiagen GmbH), High Pure Viral Nucleic Acid Kit (Roche Diagnostics, EUA), Invisorb Spin Blood Midi Kit (Invitex GmbH), and DBS Genomic DNA Isolation Kit (Norgen Biotek Corporation). Two extraction methods were recommended for DNA extraction in dried blood samples (Qiagen and Norgen extraction kits), and there is no manufacturer information for the other assays (Roche and Invitex extraction kits). To standardize the comparison of the methods, the same number of punched-out circles (three circles of 3 mm) and the same final volume of extracted DNA elution (50  $\mu$ l) were used for all methods. We followed the same volume of elution buffer as described by Lira et al. (2009; 50  $\mu$ l) of the eluate.

For the QIAamp<sup>®</sup> DNA Blood Mini Kit, the DBS protocol was followed, where three punched-out circles (3 mm) of DBS were used. The manufacturer's instructions were followed, but the volume of elution buffer was decreased (50  $\mu$ l of elution buffer was used instead of 150  $\mu$ l).

The High Pure Viral Nucleic Acid Kit (Roche Diagnostics, EUA) is recommended for isolating viral DNA and RNA from several sample materials, including serum, plasma, whole blood, and cell culture supernatant, but there is no recommendation for DBS. A total of three circles of 3 mm DBS were used, instead of 200  $\mu$ l of serum. DBS was directly used in DNA extraction, and its first step involved cell lysis by proteinase K. After this, the liquid containing nucleic acids was transferred to a silica membrane and the DNA material was eluted from the silica membrane with 50  $\mu$ l of elution buffer as recommended by the manufacturer's instructions.

Some changes were made to extract the DNA from the DBS with the Invisorb Spin Blood Midi (Invitex GmbH). As this kit is used to extract DNA from whole blood, the manufacturers suggest the first step to lysis erythrocytes and to produce a lymphocyte pellet. This step was not conducted, considering that lymphocyte cells were not our goal for DNA extraction and to prevent the loss of viral material from DBS. All the other steps were performed as recommended by the manufacturer's instructions. Three punched-out circles (3 mm) of DBS were used with 50  $\mu$ l of elution buffer, instead of the 200  $\mu$ l recommended by the manufacturer.

The DBS Genomic DNA Isolation Kit (Norgen Biotek Corporation) was the only extraction method recommended specifically to extract DNA from DBS. A total of three circles of 3 mm DBS and 50  $\mu$ l

TABLE 1 DNA concentration and purity determined by spectrophotometric analysis according to filter paper and extraction methods

Extraction method	Whatman 903 paper		Whatman FTA cards	
	DNA concentration mean $\pm$ SD (95% CI) ng/ $\mu$ l	Ratio A260 nm/A280 nm $\pm$ SD (95% CI)	DNA concentration mean $\pm$ SD (95% CI) ng/ $\mu$ l	Ratio A260 nm/A280 nm $\pm$ SD (95% CI)
QIAamp <sup>®</sup> DNA Blood Mini Kit (Qiagen GmbH)	1.82 $\pm$ 0.74 (1.20–2.45)	1.54 $\pm$ 0.67 (0.97–2.10)	2.16 $\pm$ 0.82 (1.47–2.85)	0.82 $\pm$ 0.20 (0.65–0.99)
High Pure Viral Nucleic Acid Kit (Roche Diagnostics)	223.54 $\pm$ 25.69 (202.06–245.02)	3.17 $\pm$ 0.10 (3.07–3.25)	217.56 $\pm$ 31.52 (191.20–243.92)	3.17 $\pm$ 0.11 (3.07–3.27)
Invisorb Spin Blood Midi Kit (Invitex GmbH)	9.68 $\pm$ 1.9 (8.09–11.28)	3.37 $\pm$ 0.67 (2.90–4.01)	32.93 $\pm$ 11.83 (23.04–42.83)	0.47 $\pm$ 0.12 (0.36–0.57)
Dried Blood Spot (DBS) Genomic DNA Isolation Kit (Norgen Biotek Corporation)	7.07 $\pm$ 1.89 (5.48–8.66)	1.50 $\pm$ 0.22 (1.35–1.71)	5.27 $\pm$ 1.44 (4.06–6.48)	1.63 $\pm$ 0.23 (1.43–1.82)
<i>p</i> -value	<0.0001	0.0004	<0.0001	<0.0001

A total of nine samples were used to evaluate each method. CI, confidence interval. Kruskal–Wallis with post-test (ANOVA) was used; SD, Standard deviation.

of elution buffer were used instead of the recommended 200  $\mu$ l of elution buffer.

The eluted DNA (50  $\mu$ l) was quantified by spectrophotometry (NanoDrop<sup>®</sup> 2000, Thermo Scientific). DNA volume used in the PCR reactions was defined based on DNA quantification. The 260/280 ratio of the DNA samples was also calculated to determine sample purity.

### 2.3 | HBV DNA qualitative detection

HBV DNA qualitative detection was performed by two PCR assays with PCR conditions previously described. One employed the oligonucleotides for the core region of HBV and produces a 441 base pair (bp) DNA fragment (Oliosio et al., 2007; Portilho et al., 2012), while the other uses oligonucleotides for the polymerase and surface (S/pol) region, generating a DNA fragment of approximately 1000 bp (Mallory et al., 2011). Recommended PCR conditions were followed in this study without modifications.

### 2.4 | HBV quantitative detection

HBV DNA quantification was made using the TaqMan<sup>®</sup> assay and oligonucleotides for the pre-S2/S region as previously described by Portilho et al. (2018). Briefly, a synthetic curve with a viral load, ranging from  $3 \times 10^1$  copies/ml to  $3 \times 10^7$  copies/ml, was used as an HBV standard PCR template, and a real-time reaction was run on a Rotor-Gene Q Series instrument (Qiagen). The analysis was performed using the Rotor-Gene Q Series software (Qiagen). The HBV standard contained 82 bp of the pre-S1/S2 region of HBV and was synthesized by IDT<sup>®</sup>. DNA samples extracted from each paper and each kit tested were analyzed in duplicate. The cycle threshold (CT) values obtained were compared with the expected viral load from serial dilution panel samples. Values greater than 40 were not considered, as well as those results with amplification in only one of the duplicate samples.

### 2.5 | Statistical analysis

Descriptive analyses were performed using mean, standard deviation, frequencies, and 95% confidence intervals (CI). A value of  $p < 0.05$  was considered significant. To compare continuous variables, Kruskal–Wallis with post-test (ANOVA) was used. Statistical analysis was performed using GraphPad InStat 3.01 (GraphPad Software).

## 3 | RESULTS

### 3.1 | Viral DNA extraction evaluation by DNA quality

The highest total DNA concentration was obtained in Whatman 903 paper samples extracted using the High Pure Viral Nucleic Acid Kit, Roche Diagnostics. Regarding purity, all samples presented an A260/A280 ratio above 1.5, which is considered appropriate for DNA extraction, except for DNA extracted from Whatman FTA cards using the QIAamp<sup>®</sup> DNA Blood Mini Kit and the Invisorb Spin Blood Midi Kit. An A260/A280 ratio above 3.0 could be the result of RNA coextraction, and this was observed using the High Pure Viral Nucleic Acid Kit (Roche Diagnostics) along with Whatman FTA cards or Whatman 903 paper and the Invisorb Spin Blood Midi Kit using Whatman 903 paper. A statistically significant difference was found between the DNA purity of the Whatman 903 paper and Whatman FTA cards using all methods evaluated with better results when Whatman 903 paper was used (Table 1).

### 3.2 | PCR assays for the qualitative detection of HBV

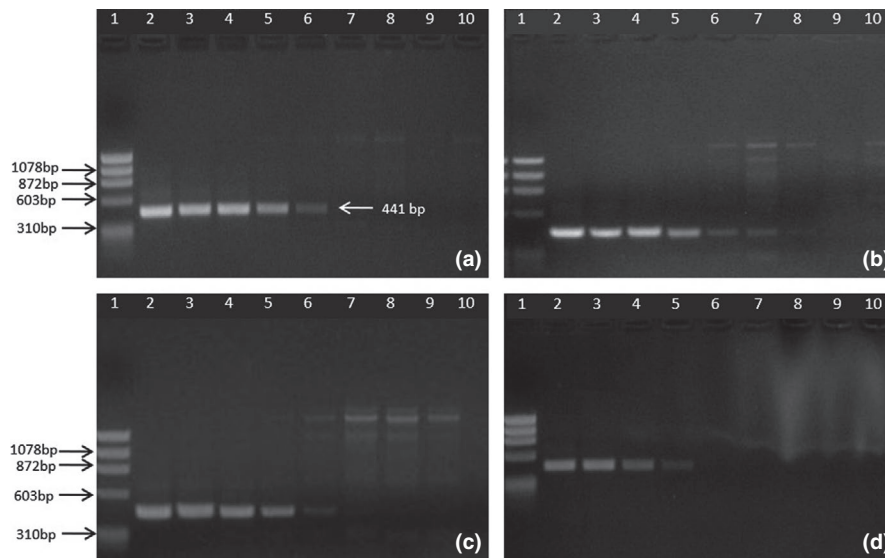
In the case of qualitative PCR, the lowest limit of detection was observed using Roche extraction (estimated at  $2 \times 10$  copies/ml). The

limit of detection was  $2 \times 10^3$ ,  $2 \times 10^3$ , and  $2 \times 10^4$  copies/ml for Qiagen, Invitex, and Norgen extraction kits, respectively (Figure 1).

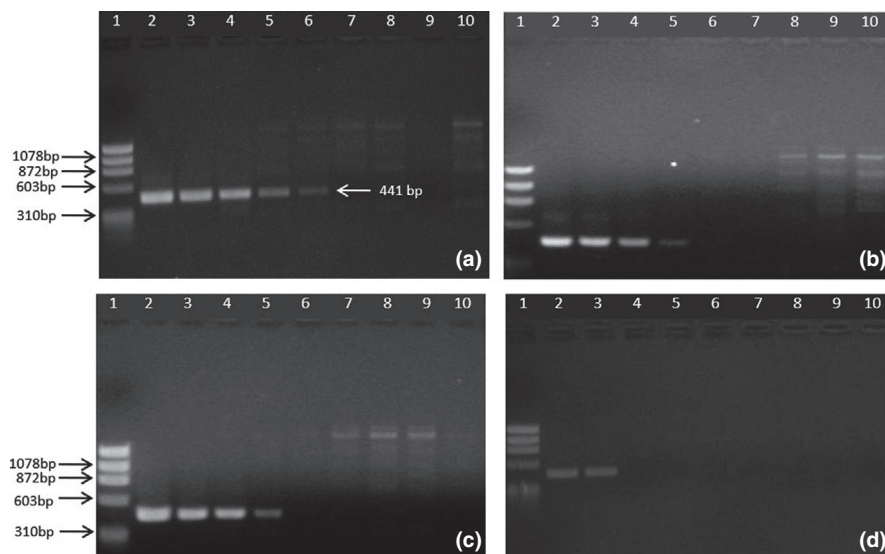
Using Whatman FTA cards and PCR to amplify the HBV core gene, the lowest limit of detection was found using samples extracted with the QIAamp<sup>®</sup> DNA Blood Mini Kit (Qiagen;  $2 \times 10^3$  copies/ml), while the highest limit of detection was observed using the DBS Genomic DNA Isolation Kit (Norgen;  $2 \times 10^6$  copies/ml; Figure 2). The limits of detection of the High Pure Viral Nucleic Acid

Kit (Roche Diagnostics) and Invisorb Spin Blood Midi Kit (Invitex) were  $2 \times 10^4$  copies/ml. Nonspecific bands were observed when samples were extracted using the QIAamp<sup>®</sup> DNA Blood Mini Kit and High Pure Viral Nucleic Acid Kit.

Using Whatman 903 paper, PCR for the surface/polymerase region of HBV gave the lowest limit of detection using the High Pure Viral Nucleic Acid Kit (2 copies/ml) and the highest limit of detection using the DBS Genomic DNA Isolation Kit (Norgen) ( $2 \times 10^4$  copies/ml).



**FIGURE 1** Agarose gel electrophoresis (1% agarose gel) of PCR products obtained for HBV core gene and Whatman 903 paper. From (a) to (d): Line 1:  $\phi$ x174 DNA-Hae III Digest, as molecular standard. Lanes 2–9: Ten-fold serial dilutions of HBV serum samples in whole blood spotted on Whatman 903 paper (ranging from  $2 \times 10^7$  to 2 copies/ml). Line 10: DBS without HBV as a negative control. (a) DBS samples extracted with the QIAamp DNA Mini Kit. (b) DBS samples extracted with the High Pure Viral Nucleic Acid Kit. (c) DBS samples extracted with the Invisorb Spin Blood Midi Kit. (d) DBS samples extracted with the DBS Genomic DNA Isolation Kit



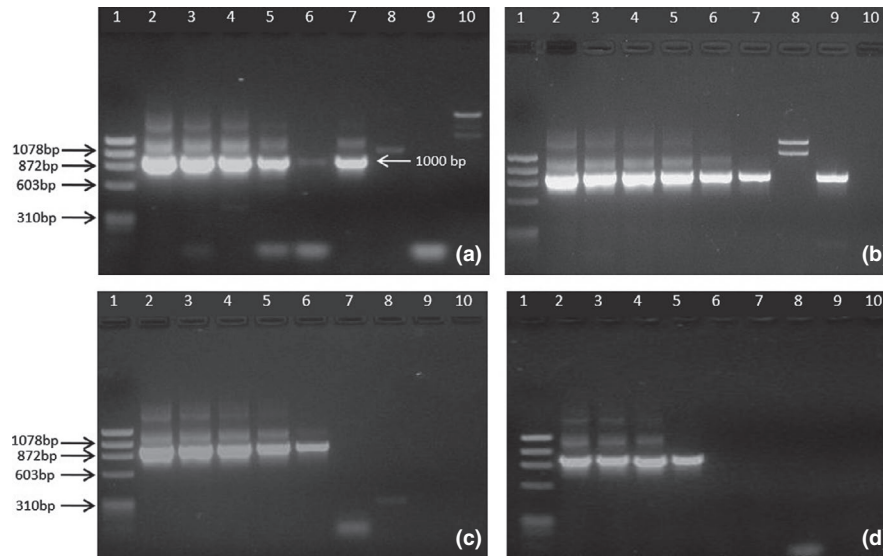
**FIGURE 2** Agarose gel electrophoresis (1% agarose gel) of PCR products obtained for the core gene of HBV and Whatman FTA cards. (From (a) to (d): Line 1:  $\phi$ x174 DNA-Hae III Digest, as molecular standard. Lanes 2–9: Ten-fold serial dilutions of HBV serum samples in whole blood spotted on Whatman 903 paper (ranging from  $2 \times 10^7$  to 2 copies/ml). Line 10: DBS without HBV as a negative control. (a) DBS samples extracted with the QIAamp DNA Mini Kit. (b) DBS samples extracted with the High Pure Viral Nucleic Acid Kit. (c) DBS samples extracted with the Invisorb Spin Blood Midi Kit. (d) DBS samples extracted with the DBS Genomic DNA Isolation Kit

The limits of detection of the QIAamp® DNA Blood Mini Kit (Qiagen) and Invisorb Spin Blood Midi Kit (Invitex) were  $2 \times 10^2$  and  $2 \times 10^3$  copies/ml, respectively (Figure 3).

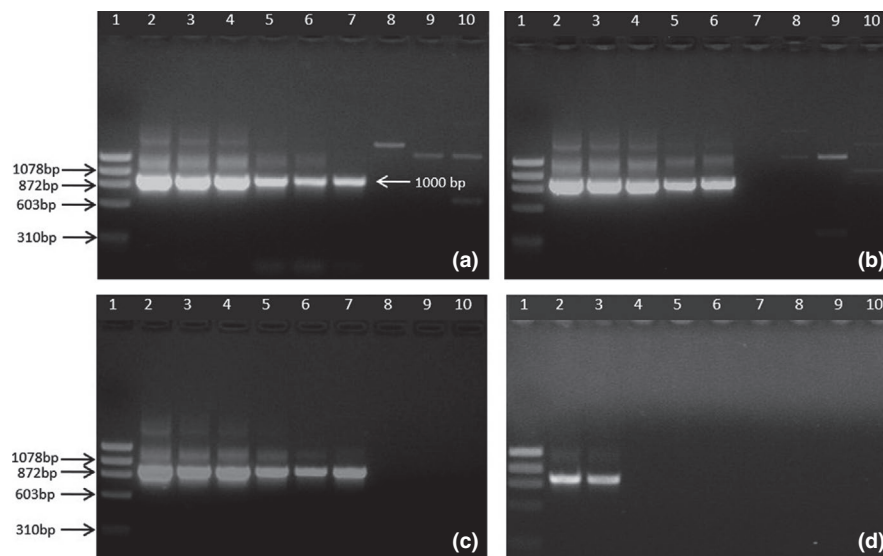
Using Whatman FTA cards, PCR for the surface/polymerase region of HBV gave the lowest limit of detection using the QIAamp® DNA Blood Mini Kit (Qiagen) and Invisorb Spin Blood Midi Kit (Invitex;  $2 \times 10^2$  copies/ml). The lowest limits of detection for the

High Pure Viral Nucleic Acid Kit and DBS Genomic DNA Isolation Kit were  $2 \times 10^3$  and  $2 \times 10^6$  copies/ml, respectively (Figure 4).

DBS (Whatman 903 paper) and serum samples from 10 individuals (five HBV-infected and five uninfected) were tested using the High Pure Viral Nucleic Acid Kit and PCR for the surface/polymerase region of HBV and presented concordant results in all samples.



**FIGURE 3** Agarose gel electrophoresis (1% agarose gel) of PCR products obtained for surface/polymerase gene of HBV and Whatman 903 paper. From (a) to (d): Line 1:  $\phi$ x174 DNA-Hae III Digest, as molecular standard. Lanes 2–9: Ten-fold serial dilutions of HBV serum samples in whole blood spotted on Whatman 903 paper (ranging from  $2 \times 10^7$  to 2 copies/ml). Line 10: DBS without HBV as a negative control. (a) DBS samples extracted with the QIAamp DNA Mini Kit. (b) DBS samples extracted with the High Pure Viral Nucleic Acid Kit. (c) DBS samples extracted with the Invisorb Spin Blood Midi Kit. (d) DBS samples extracted with the DBS Genomic DNA Isolation Kit



**FIGURE 4** Agarose gel electrophoresis (1% agarose gel) of PCR products obtained for surface/polymerase gene of HBV and Whatman FTA cards. From (a) to (d): Line 1:  $\phi$ x174 DNA-Hae III Digest, as molecular standard. Lanes 2–9: Ten-fold serial dilutions of HBV serum samples in whole blood spotted on Whatman 903 paper (ranging from  $2 \times 10^7$  to 2 copies/ml). Line 10: DBS without HBV as a negative control. (a) DBS samples extracted with the QIAamp DNA Mini Kit. (b) DBS samples extracted with the High Pure Viral Nucleic Acid Kit. (c) DBS samples extracted with the Invisorb Spin Blood Midi Kit. (d) DBS samples extracted with the DBS Genomic DNA Isolation Kit



### 3.3 | HBV DNA quantitative detection

An in-house real-time PCR (qPCR) was used to detect and quantify HBV DNA in DBS. Table 2 shows the mean CT values observed between the duplicates for each point of the dilution panel. The lowest limit of detection was found using Whatman 903 paper and the QIAamp<sup>®</sup> DNA Blood Mini Kit or High Pure Viral Nucleic Acid Kit ( $2 \times 10^3$  copies/ml). The CT values observed in Whatman 903 paper or Whatman FTA card samples extracted using the QIAamp<sup>®</sup> DNA Blood Mini Kit or High Pure Viral Nucleic Acid Kit demonstrated that these extraction methods were more appropriate for quantitative detection of HBV. All samples presenting the viral load of more than  $2 \times 10^6$  copies/ml were detected, regardless of the extraction kit or filter paper used. Using Whatman FTA cards, it was possible to detect HBV DNA in samples with  $2 \times 10^4$  copies/ml by three of the four evaluated methods (QIAamp<sup>®</sup> DNA Blood Mini Kit, High Pure Viral Nucleic Acid Kit, Invisorb Spin Blood Midi Kit).

DBS (Whatman 903 paper) and serum samples from 10 individuals (five HBV-infected and five uninfected) were tested using the

High Pure Viral Nucleic Acid Kit and real-time PCR and gave 100% concordant results compared to serum tested with the commercial method (HBV Abbott). The mean  $\pm$  SD of CT values of HBV-infected individuals was  $36.2 \pm 0.4$  in DBS and  $34.9 \pm 1.6$  in serum using in-house real-time PCR.

## 4 | DISCUSSION

HBV infection is a major public health problem, with more than 200 million people chronically infected, most of them unaware of their status. Alternative samples such as DBS could be useful in increasing diagnostic access in different settings. For this reason, developing a suitable method for detecting HBV DNA is essential. However, due to a filter paper, identifying an efficient method of DNA extraction for HBV detection and quantification by PCR is a real challenge. This is the first study comparing different methods to find a reliable and efficient method for HBV DNA detection in DBS.

TABLE 2 Mean CT values determined by the qPCR test according to the filter paper and extraction method used

Viral load (copies/ml)	Whatman 903 paper				p-value
	T1	T2	T3	T4	
	Mean <sup>a</sup> $\pm$ SD of CT values (95% CI)	Mean <sup>a</sup> $\pm$ SD of CT values (95% CI)	Mean <sup>a</sup> $\pm$ SD of CT values (95% CI)	Mean <sup>a</sup> $\pm$ SD of CT values (95% CI)	
$2 \times 10^7$	19.44 $\pm$ 0.11 (18.48–20.38)	20.43 $\pm$ 0.11 (19.41–21.44)	21.79 $\pm$ 0.30 (19.05–24.51)	25.51 $\pm$ 0.35 (22.39–28.61)	<0.0001
$2 \times 10^6$	23.60 $\pm$ 0.07 (22.96–24.23)	24.14 $\pm$ 0.05 (23.69–24.58)	25.25 $\pm$ 0.25 (22.96–27.53)	29.65 $\pm$ 0.09 (28.81–30.47)	<0.0001
$2 \times 10^5$	25.95 $\pm$ 0.16 (24.55–27.34)	26.99 $\pm$ 0.18 (25.39–28.57)	27.52 $\pm$ 0.00 (27.52–27.52)	31.81 $\pm$ 0.09 (30.92–32.69)	0.038
$2 \times 10^4$	29.67 $\pm$ 0.14 (28.39–30.94)	30.60 $\pm$ 0.79 (23.48–37.71)	31.15 $\pm$ 0.93 (22.82–39.46)	34.92 $\pm$ 0.74 (28.24–41.58)	0.038
$2 \times 10^3$	33.18 $\pm$ 0.87 (25.36–40.89)	34.57 $\pm$ 0.59 (29.23–39.90)	ND	33.62 $\pm$ 0.00 (33.62–33.62) ND	0.33
$2 \times 10^2$	ND	ND	ND	ND	–
$2 \times 10^1$	ND	ND	ND	ND	–
$2 \times 10^0$	ND	ND	ND	ND	–
Viral load (copies/ml)	Whatman FTA cards				p-value
	T1	T2	T3	T4	
	Mean <sup>a</sup> CT values ( $\pm$ SD)	Mean <sup>a</sup> CT values ( $\pm$ SD)	Mean <sup>a</sup> CT values ( $\pm$ SD)	Mean <sup>a</sup> CT values ( $\pm$ SD)	
$2 \times 10^7$	21.16 $\pm$ 0.01 (21.03–21.28)	23.13 $\pm$ 0.01 (23.00–23.25)	26.13 $\pm$ 0.01 (26.00–26.25)	32.10 $\pm$ 0.13 (30.89–33.31)	0.0095
$2 \times 10^6$	25.08 $\pm$ 0.28 (22.59–27.55)	26.27 $\pm$ 0.06 (25.76–26.77)	30.63 $\pm$ 0.01 (30.50–30.75)	34.56 $\pm$ 0.25 (32.33–36.77)	0.0095
$2 \times 10^5$	29.02 $\pm$ 0.49 (24.63–33.39)	29.84 $\pm$ 0.55 (24.88–34.79)	33.62 $\pm$ 0.04 (33.23–34.00)	ND	0.06
$2 \times 10^4$	32.39 $\pm$ 0.28 (29.90–34.86)	33.51 $\pm$ 0.57 (28.42–38.59)	37.29 $\pm$ 0.64 (31.50–43.06)	ND	0.06
$2 \times 10^3$	ND	ND	ND	ND	–
$2 \times 10^2$	ND	ND	ND	ND	–
$2 \times 10^1$	ND	ND	ND	ND	–
$2 \times 10^0$	ND	ND	ND	ND	–

T1, QIAamp<sup>®</sup> DNA Blood Mini Kit (Qiagen GmbH, Germany); T2, High Pure Viral Nucleic Acid Kit (Roche Diagnostics, EUA); T3, Invisorb Spin Blood Midi Kit (Invitex GmbH, Berlin); T4, Dried Blood Spot (DBS) Genomic DNA Isolation Kit (Norgen Biotek Corporation, Canada); SD, Standard deviation; CT, cycle threshold; CI, confidence interval; ND, Not detected.

<sup>a</sup>n = 2. The tests were conducted in duplicate to give viral load.

All extraction methods enabled us to extract enough DNA from DBS for molecular analysis. Regarding purity, Whatman 903 paper produced superior results. Although all samples presented an appropriate A260/A280 ratio, mean ratio values above 3.0 were observed using the High Pure Viral Nucleic Acid Kit (Roche Diagnostics). Considering that this extraction kit, according to the manufacturer, was designed to extract total nucleic acids from the sample, these ratios could be the result of RNA coextraction. Indeed, the detection of an RNA virus from DBS using this extraction method has been shown previously (Monleau et al., 2009).

Qualitative HBV DNA detection was more appropriate using Whatman 903 paper along with the Roche Extraction method. Many studies have used Whatman 903 paper for PCR diagnosis of infectious diseases, validated by the Food and Drugs Administration (FDA), which only recommends the Whatman 903 and Perkin Elmer ([Beaconsfield, UK] 226 filter papers) papers for collecting human blood (Aitken et al., 2015; Duncombe et al., 2013; Gupta et al., 1992; Jardi et al., 2004; Lira et al., 2009; Masciotra et al., 2012; Smit et al., 2014). Previous studies found sensitivity from 88 to 100% for HBV DNA quantitative detection using Whatman 903 paper (Alhuseini et al., 2012; Stene-Johansen et al., 2016).

Core and S/pol regions were evaluated for detecting HBV DNA in qualitative PCR. These two HBV targets are frequently used in commercial assays to detect HBV in serum (Abbott RealTime HBV Assay and the Cobas TaqMan HBV test, version 2; Liu et al., 2019) and are employed for detecting occult HBV or acute cases (Liu et al., 2019; Sosa-Jurado et al., 2018).

Quantitative HBV detection had better results using QIAamp® DNA Blood Mini Kit (Qiagen) and High Pure Viral Nucleic Acid Kit (Roche Diagnostics) and Whatman 903 paper ( $2 \times 10^3$  copies/ml) compared to Whatman FTA cards ( $2 \times 10^4$  copies/ml). Jardi et al. (2004) also found a limit of  $2 \times 10^3$  copies/ml in DBS using QIAamp® DNA Blood Mini Kit (Qiagen). Halfon et al. (2012) improved this using a commercial quantification technique and obtained a limit of detection of  $8.148 \times 10^3$  copies/ml. This study, through a systematic analysis of the three variables, lowered this further and achieved a limit of detection of  $2 \times 10^3$  copies/ml through the combined use of Whatman 903 paper along with the QIAamp® DNA Blood Mini Kit (Qiagen) or High Pure Viral Nucleic Acid Kit (Roche Diagnostics) and in-house real-time PCR.

The High Pure Viral Nucleic Acid Kit (Roche Diagnostics) has been used for HIV RNA in dried plasma, but this is the first report of this assay for detecting HBV DNA in DBS (Monleau et al., 2009). In the case of real-time PCR and the Invisorb Spin Blood Midi Kit (Invitex), false-negative results for detecting the sample containing  $2 \times 10^5$  copies/ml were observed, which could be the result of an inhibitor present in this sample that cannot be removed by this method.

The main objective of this study is to provide new information on HBV DNA detection in DBS samples and an optimized protocol for the extraction and detection of HBV DNA in these samples. This information will be helpful in the application of future large-scale studies. However, there are some limitations, such as a small number of samples from individuals included in the optimized method

evaluation, the absence of manual DNA extraction methods, and lack of internal control.

In conclusion, Whatman 903 paper along with QIAamp® DNA Blood Mini Kit (Qiagen) or High Pure Viral Nucleic Acid Kit (Roche Diagnostics) extraction kits presented high sensitivity for qualitative and quantitative HBV DNA detection. These procedures will be useful tools to increase molecular HBV diagnostic access in remote areas where cold storage and transportation are difficult.

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## CONFLICT OF INTEREST

None declared.

## AUTHOR CONTRIBUTIONS

**Cristianne Sousa Bezerra:** Conceptualization (equal); Investigation (equal); Methodology (lead); Writing-original draft (equal); Writing-review & editing (equal). **Moyra Machado Portilho:** Conceptualization (equal); Investigation (equal); Methodology (equal); Writing-original draft (equal). **Cristiane Cunha Frota:** Conceptualization (equal); Data curation (equal); Funding acquisition (lead); Writing-original draft (equal); Writing-review & editing (equal). **Livia Melo Villar:** Investigation (equal); Methodology (equal); Writing-original draft (equal); Writing-review & editing (lead).

## ETHICS STATEMENT

Protocols and procedures of this study were reviewed and approved by Fiocruz Ethics Committee (approval number: 889.582). Informed consent was obtained from all participants who gave blood samples.

## DATA AVAILABILITY STATEMENT

All data are provided in full in the results section of this article.

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