A comparison of molecular methods for hepatitis B virus (HBV) DNA detection from oral fluid samples

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The objective of the present study was to evaluate four commercial DNA extraction methods and three PCR protocols for hepatitis B virus (HBV) detection in artificially contaminated oral fluid samples. The extraction protocols were selected based on ease of use and cost, and were also compared with respect to sensitivity and cost. Prior PCR optimization was conducted, in which the sample volume for DNA extraction and the concentrations of DNA and Taq DNA polymerase in the PCR were adjusted. One-round PCR, used to amplify the core region of the HBV genome, achieved high levels of sensitivity in comparison with nested and semi-nested PCR experiments that were designed for the amplification of HBV surface protein genes. Of the four extraction protocols evaluated, the RTP DNA/RNA Virus Mini kit and the QIAamp DNA Mini kit gave the highest recovery rates, presenting 20 copies of HBV DNA ml$^{-1}$ as the limit of detection. These results suggest that HBV DNA can be detected from oral fluid samples but that the optimization of the PCR assays and the choice of extraction methods must be determined by laboratories before the implementation of this method in routine diagnostics.

INTRODUCTION

Hepatitis B virus (HBV) attacks the liver and can cause acute or chronic infection. Approximately two billion people worldwide have been infected with the virus, and approximately 350 million live with chronic infection (WHO, 2008). The virion is composed of a nucleocapsid that is covered by envelope proteins. In the nucleocapsid, there is an incomplete dsDNA and a DNA polymerase (Seeger & Zoulim, 2007). Real-time and traditional PCR methods are useful for the detection and quantification of infectious agents in clinical specimens (Olive & Bean, 1999; Clarke, 2002; Metwally et al., 2008). These methods can be employed to decide if establishment of HBV treatment is necessary and to monitor the efficacy of that treatment (Dai et al., 2004; Lu et al., 2006).

The extraction of a sufficient quantity of nucleic acid, combined with the removal of substances that are inhibitory to amplification, is pivotal to the optimal detection of microbial pathogens by PCR (McOrist et al., 2002; Read, 2001). The failure to remove enzymic inhibitors or to extract sufficient nucleic acids could result in the inappropriate categorization of a specimen as falsely negative for a pathogen (Honore-Bouakline et al., 2003; Smith et al., 2003). Therefore, nucleic acid extraction is an important initial step in molecular diagnostics. Recently, many kits for the extraction of nucleic acids from various types of clinical specimens, such as blood and oral fluid, have been introduced and are commercially available (Durdiaková et al., 2012; Lee et al., 2010; Metwally et al., 2008; Smith et al., 2003; Viltrop et al., 2010).

Although serum specimens are the principal choice for the detection of the viral components of hepatitis B infection, as well as other infectious diseases, it has been demonstrated that DNA extracted from oral fluid and other clinical specimens could yield DNA of equivalent quality to that extracted from the blood and, thereby, could serve as a reliable alternative DNA source for this purpose (Karayiannis et al., 1985; Hutse et al., 2005; van der Eijk et al., 2005; Amado et al., 2006; Kidd-Ljunggren et al., 2006). It has been shown that, when the HBV viral load is elevated in the serum, the same effect occurs in the oral fluid (Zhang et al., 2008), making it possible for the transmission and diagnosis of infection to occur by this route; the virus is also present in the oral fluid of HBsAg- and HBeAg-positive patients (Noppornpanth et al., 2000; Heiberg et al., 2010).

There are many advantages to using oral fluid for HBV DNA detection. Oral fluid collection is less invasive and presents a lower risk of disease transmission compared with blood collection. For these reasons, oral fluid samples have been studied as an alternative for HBV detection (Zhevachevsky et al., 2000; Hutse et al., 2005; Cruz et al., 2011). However, although HBV DNA has been detected in oral fluid samples (Heiberg et al., 2010), an established protocol for HBV DNA extraction and detection from such samples is still needed.

Abbreviations: HAV, hepatitis A virus; HBV, hepatitis B virus.
In the present study, four methods for DNA extraction from oral fluid samples were compared: (i) the QIAamp DNA Mini kit (Qiagen); (ii) the High Pure PCR Template Preparation kit (Roche Diagnostics); (iii) the RTP DNA/RNA Virus Mini kit (Invitek); and (iv) the Saliva DNA Isolation kit (Norgen Biotek Corporation). These methods were tested on the same samples with three protocols for qualitative detection using specific primers for different regions of the HBV genome. The goal of this study was to optimize the molecular methods for HBV DNA detection from oral fluid samples.

**METHODS**

**Specimens.** The study was carried out from March 2010 to July 2011, after obtaining Fiocruz Ethics Committee Approval (protocol number 433/07). Oral fluid samples were obtained from 20 healthy adult volunteers without serological evidence of HBV infection (absence of HBsAg and anti-HBe markers). These volunteers were health professionals at Oswaldo Cruz Institute and gave their written informed consent to participate in this study.

The oral fluid samples were obtained using a commercial collector (Salivette, Sarstedt) according to the manufacturer’s instructions. After collection, 1 ml PBS (pH 7.2) was added to minimize the effects of the degradation of the oral fluid and to facilitate pipetting. The vials were centrifuged at 2000 g for 10 min and 1 ml oral fluid was extracted and stored at −20 °C. The oral fluid samples were also checked visually for blood contamination. If contamination occurred, the samples were excluded. However, no contamination occurred in this study because all of the samples were taken properly.

The oral fluid samples obtained were pooled, and two aliquots were prepared. One aliquot was used as a negative control for the extraction and detection reactions, and the other was serially spiked with a commercial positive control containing HBV DNA (OptiQuant, Acrometrix). The oral fluid samples without the commercial HBV standard were tested to exclude the possibility that the pool contained a low level of HBV DNA that would be variably detected by PCR.

The commercial HBV standard contains 2 × 10^7 copies of HBV ml^-1 and was serially spiked in a 10-fold serial dilution on oral fluid samples, giving HBV concentrations ranging from 2 × 10^6 to 2 × 10^-2 copies ml^-1. These samples were employed to evaluate the analytical sensitivity.

**Viral DNA extraction.** HBV DNA was isolated from each sample using one of four commercial methods. All of the assays followed adsorption elution on a silica membrane and were conducted according to the manufacturer’s instructions, with slight modifications in the sample and elution volumes for certain assays. Three assays can be employed for DNA extraction from oral fluid samples, according to suppliers (the Saliva DNA Isolation kit, the RTP DNA/RNA Virus Mini kit and the QIAamp DNA Mini kit); however, the sample collection method described for these assays was not the same method that was employed in the present study.

The Saliva DNA Isolation kit (Norgen Biotek Corporation) contains a preservation solution and is designed for oral fluid samples collected by spitting or swabbing. In the present study, 500 µl of each oral fluid sample was mixed with 500 µl of the preservation solution, according to the manufacturer’s instructions, and 500 µl of this mixture was employed. Subsequently, 100 µl of elution buffer was used, which is the amount recommended in the kit instructions.

For the QIAamp DNA Mini kit (Qiagen), the ‘Buccal Swab Spin Protocol’ was followed. This protocol was designed for cotton swabs, Dacron swabs or Omni swabs. The sample volume was increased twofold relative to the manufacturer’s recommendations (400 µl instead of 200 µl), although the recommended volume of elution buffer was used (150 µl).

For the RTP DNA/RNA Virus Mini kit (Invitek), it is normally necessary to add 400 µl of ddH2O to a collection swab; however, in the present study, 400 µl of oral fluid obtained with a Salivette collector was used, along with the recommended volume of elution buffer (60 µl), to obtain the DNA.

In the High Pure PCR Template kit (Roche Diagnostics), no change was made to the initial sample volume, so 200 µl of oral fluid was used. However, the DNA was eluted in 100 µl of elution buffer instead of the 200 µl recommended by the manufacturer.

All of the extraction methods were repeated three times and each time, triplicates of the nine HBV concentrations were extracted. To prevent PCR cross-contamination, separate rooms were used to perform the PCR procedures, including the nucleic acid extraction, the preparation of the PCR reagents, the setting up of the PCR experiments, the PCR amplification and the post-PCR analysis. Positive and multiple negative controls were included in each PCR run. The human β globin gene was the internal PCR control and was tested as previously described (Lee et al., 2010).

**HBV DNA PCR**

**Oligonucleotides.** Oligonucleotides specific for the core and surface regions of the HBV genome were employed for the following PCR experiments (Table 1): a semi-nested PCR amplifying HBV surface protein genes (Naito et al., 2001) with slight modifications performed in the present study to generate a product of 1100 bp; a nested PCR for the surface regions of the HBV genome (developed in the present study) with a product of 542 bp; and a one-round PCR specific for the core region of HBV (Olioso et al., 2007), which generated a product of 441 bp.

**Optimization of the protocol.** To increase the HBV DNA detection from oral fluid samples, several PCR parameters were evaluated using spiked HBV oral fluid samples that had the DNA extracted with the QIAamp DNA Mini kit and that were detected using semi-nested PCR (Naito et al., 2001). The QIAamp DNA Mini kit has been used in other studies for DNA extraction from saliva samples and exhibited good efficiency (Viltrop et al., 2010; Durdiaková et al., 2012), so this assay was used in the initial experiments for the optimization of the PCR conditions. After PCR optimization, this assay was applied to the other extraction methods and the limit of sensitivity was defined for each extraction method.

The Platinum Taq DNA polymerase (Invitrogen) concentration (5 U µl^-1), using 0.1 µl (0.5 U) and 0.3 µl (1.5 U); the DNA volume, at 2 µl and 5 µl; the annealing temperature of the primers, at 47 °C and 55 °C; the number of amplification cycles (30, 35 and 40 cycles); and the time of extension of the DNA chain (1 and 2 min) were evaluated. After optimization, this protocol was employed for nested PCR.

**PCR conditions.** Semi-nested PCR was performed with Platinum Taq DNA polymerase (Invitrogen) to amplify 1100 bp from the pre-S/S region. In the first round, this assay uses the sense primer PS1 and a mixture of the antisense primers S2 and S22, which permits the amplification of all HBV genotypes. The second round of amplification was performed on the entire first-round PCR product, using the primers PS1 and SR (Naito et al., 2001).

The first PCR was carried out in a tube containing 25 µl reaction buffer made up of the following components: 0.2 µM each primer,
0.2 mM each of the four deoxynucleotides, 10 × PCR buffer, 3.0 mM MgCl₂, 0.5 or 1.5 U Platinum Taq polymerase (Invitrogen), and 2 or 5 μl target DNA. A negative control reaction tube received 25 μl of the reaction mixture, without target DNA. Positive controls were included for each target tested. The thermocycler (T3 Thermocycler, Biometra) was programmed to first incubate the samples for 3 min at 95 °C; followed by 35 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min; and a final extension step of 72 °C for 7 min. The number of cycles (35 or 40) and the annealing temperature of the primers (55 °C or 47 °C) were also evaluated in this procedure, as described above.

A second round of PCR was performed using a 2 μl aliquot of the first PCR product. The second sets of each of the inner primer pairs (PS1 and SR) were used at 0.3 μM, and each of the deoxynucleotides, the Platinum Taq polymerase, and the PCR buffer were used as in the first reaction. The cycling conditions of the reaction were as follows: 94 °C for 3 min; followed by 40 cycles of 95 °C for 30 s, 52 °C for 40 s, and 72 °C for 2 min; and a final extension at 72 °C for 7 min. The number of cycles (30 or 40), the Taq DNA polymerase concentration (0.5 or 1.5 U), and the time of DNA extension (1 or 2 min) were also evaluated in this procedure, as described above.

The nested PCR was performed using the same reagents and optimized conditions as described above for the semi-nested PCR; this procedure used the primers PS1, S2 and S22 for the first round of amplification and the sense primer S1 and anti-sense primer SR for the second round of amplification.

One-round PCR to amplify the core region of the HBV genome (Olioso et al., 2007) was conducted using 0.2 μM each primer, 0.2 mM each of the four deoxynucleotides, 10 × PCR buffer, 3.0 mM MgCl₂, 1.5 U Platinum Taq polymerase and 5 μl target DNA. The PCR conditions were 94 °C for 3 min; followed by 35 cycles of 94 °C for 30 s, 62 °C for 30 s, 72 °C for 30 s, and a final extension step at 72 °C for 7 min.

Identification of the amplicon sequences. PCR products of 1100 bp, for the semi-nested PCR, 580 bp, for the nested PCR, and 446 bp, for the single-step PCR, were visualized with UV light as single bands by staining with ethidium bromide (10 mg ml⁻¹) after 1% agarose gel electrophoresis in TBE buffer.

Cost analysis. The cost per test was calculated for each assay and included the test kit, materials and reagents. The laboratory personnel salaries, equipment and laboratory overhead costs were not included in this cost. The cost per test was calculated in US dollars, based on prices obtained during the period from July to December 2010.

RESULTS

Optimization of PCR conditions

For the standardization of PCR for use on oral fluid samples, viral DNA obtained from oral fluid samples extracted with the QIAamp DNA Mini kit was first subjected to semi-nested PCR using oligonucleotides previously described by Naito et al. (2001). The best conditions for the first round of amplification were obtained when using 1.5 U Platinum Taq DNA polymerase, 5 μl DNA and 40 cycles of amplifications with a primer annealing temperature of 47 °C. For the second round of amplification, using Platinum Taq DNA polymerase at 1.5 U with 30 cycles of amplification and 1 min of extension of the DNA gave the best result.

Using semi-nested PCR without optimization, the analytical sensitivity was 2 × 10⁵ copies of HBV DNA ml⁻¹ in oral fluid samples. However, after the optimization of the PCR conditions, as described above, the detection limit of semi-nested PCR was improved to 2 × 10⁴ copies of HBV DNA ml⁻¹, as demonstrated in Fig. 1.

A nested-PCR protocol was proposed in this study, and the same reaction conditions described for the optimized semi-nested PCR were followed. The only difference between the nested PCR and the semi-nested PCR was the substitution of oligonucleotide PS1 for sense oligonucleotide S1 in the second round of amplification. Using this protocol, the limit of detection was 2 × 10³ copies of HBV DNA ml⁻¹ from oral fluid samples using DNA extracted with the QIAamp DNA Mini kit (Qiagen), which was the same limit of detection that was observed for the semi-nested PCR protocol (Fig. 2). However, smeared DNA bands were seen at a higher frequency in the semi-nested PCR than in the nested PCR (Fig. 2).

Comparison of extraction methods

After the optimization of the PCR methods, modified semi-nested PCR, nested PCR and one-round PCR (Olioso et al., 2007) were used to evaluate the four different methods of DNA extraction from the oral fluid samples. The internal control was detected in all of the oral fluid samples obtained by the extraction methods evaluated.

Using modified semi-nested PCR, the highest sensitivity could be observed on samples extracted with the QIAamp DNA Mini kit, as 20 copies ml⁻¹ were detected (Fig. 3d). Using optimized semi-nested PCR and the RTP DNA/RNA Virus Mini kit, a detection limit of 2 × 10² copies HBV DNA ml⁻¹ was observed (Fig. 3c). However, using optimized
semi-nested PCR and the High Pure PCR Template Preparation kit or the Saliva DNA Isolation kit, less satisfactory results were obtained, as $2 \times 10^3$ copies HBV DNA ml$^{-1}$ were necessary for detection (Fig. 3a and b).

Using nested PCR, the highest sensitivities were observed on DNA extracted with either the QIAamp DNA Mini kit or the RTP DNA/RNA Virus Mini kit, as 20 copies of HBV DNA ml$^{-1}$ could be detected (Fig. 4c, d). Using the nested PCR protocol and the High Pure PCR Template Preparation kit, a detection limit of $2 \times 10^2$ copies HBV DNA ml$^{-1}$ was observed (Fig. 4b). Finally, using nested PCR and the Saliva DNA Isolation kit, the results were the least satisfactory, as $2 \times 10^4$ copies of HBV DNA ml$^{-1}$ were required for detection (Fig. 4a).

Using one-round PCR (Olioso et al., 2007), the best results were obtained with the RTP DNA/RNA Virus Mini kit, with a detection limit of 20 copies of HBV DNA ml$^{-1}$, followed by the High Pure PCR Template kit and the QIAamp DNA Mini kit (with a detection limit of $2 \times 10^3$ copies HBV DNA ml$^{-1}$) and then the Saliva DNA Isolation kit (with a detection limit of $2 \times 10^5$ copies HBV DNA ml$^{-1}$) (Fig. 5).

**Cost analysis**

The cost per test of each extraction method was calculated based on the US dollar values as of August 2011. The costs of the reagents for all of the extraction methods were evaluated, and the High Pure PCR Template Preparation kit presented the lowest cost per test ($4.63), followed by the QIAamp DNA Mini kit ($5.95), the RTP DNA/RNA Virus Mini kit ($6.38), and the Saliva DNA Isolation kit ($8.67).

**DISCUSSION**

HBV DNA has been detected in oral fluid samples (van der Eijk et al., 2005; Lu et al., 2006), but there is not a standardized protocol for this purpose. Accordingly, this study was developed to standardize extraction and detection methods for HBV DNA detection in oral fluid samples. The optimization of the PCR conditions for HBV DNA detection from oral fluid samples was the primary goal of this study. First, the specificity of the PCR was determined and the primer concentration and annealing temperature were evaluated under conditions where no primer dimers were observed, although non-specific bands were present. The variable factors that were evaluated included the number of cycles, the DNA extension time and the Taq DNA polymerase concentration. Using an optimized semi-nested PCR protocol, the analytical sensitivity improved from $2 \times 10^3$ to $2 \times 10^2$ copies HBV DNA ml$^{-1}$; 11, HBV serum as a positive control; 12, oral fluid as a negative control.
Fig. 3. Agarose gel electrophoresis (1% agarose gel) of products generated by optimized semi-nested PCR of HBV DNA obtained from oral fluid samples with different extraction methods. (a) Extraction with the High Pure PCR Template Preparation kit (Roche) showing a sensitivity of $2 \times 10^3$ copies HBV DNA ml$^{-1}$. Lanes 2–5 contain positive samples. (b) Extraction with the Saliva DNA Isolation kit (Norgen), showing a sensitivity of $2 \times 10^3$ copies HBV DNA ml$^{-1}$. Lanes 2–5 contain positive samples. (c) Extraction with the RTP DNA/RNA Virus Mini kit (Invitek) showing a sensitivity of $2 \times 10^2$ copies HBV DNA ml$^{-1}$. Lanes 2–6 contain positive samples. (d) Extraction with the QIAamp DNA Mini kit (Qiagen), showing a sensitivity of $2 \times 10^1$ copies HBV DNA ml$^{-1}$. Lanes 2–7 contain positive samples. Lanes: 1, φX174, a molecular standard; 2–10, tenfold serial dilutions of HBV serum samples in oral fluid samples ($2 \times 10^6$ to $2 \times 10^{-2}$ copies HBV DNA ml$^{-1}$); 11, HBV serum as a positive control; 12, oral fluid as a negative control.

Fig. 4. Agarose gel electrophoresis (1% agarose gel) of products generated by nested PCR of HBV DNA obtained from oral fluid samples with different extraction methods. (a) Extraction with the Saliva DNA Isolation kit (Norgen) showing a sensitivity of $2 \times 10^4$ copies HBV DNA ml$^{-1}$. Lanes 2–4 contain positive samples. (b) Extraction with the High Pure PCR Template Preparation kit (Roche) showing a sensitivity of $2 \times 10^2$ copies HBV DNA ml$^{-1}$. Lanes 2–6 contain positive samples. (c) Extraction with the QIAamp DNA Mini kit (Qiagen) showing a sensitivity of $2 \times 10^1$ copies HBV DNA ml$^{-1}$. Lanes 2–7 contain positive samples. (d) Extraction with the RTP DNA/RNA Virus Mini kit (Invitek) showing a sensitivity of $2 \times 10^1$ copies HBV DNA ml$^{-1}$. Lanes 2–7 contain positive samples. Lanes: 1, φX174, a molecular standard; 2–10, tenfold serial dilutions of HBV serum samples in oral fluid samples ($2 \times 10^6$ to $2 \times 10^{-2}$ copies HBV DNA ml$^{-1}$); 11, HBV serum as a positive control; 12, oral fluid as a negative control.
Fig. 5. Agarose gel electrophoresis (1 % agarose gel) of products generated by one-round PCR of HBV DNA obtained from oral fluid samples with different extraction methods. (a) Extraction with the Saliva DNA Isolation kit (Norgen) showing a sensitivity of $2 \times 10^4$ copies HBV DNA ml$^{-1}$. Lanes 2–4 contain positive samples. (b) Extraction with the High Pure PCR Template Preparation kit (Roche) showing a sensitivity of $2 \times 10^5$ copies HBV DNA ml$^{-1}$. Lanes 2–5 contain positive samples. (c) Extraction with the QIAamp DNA Mini kit (Qiagen) showing a sensitivity of $2 \times 10^6$ copies HBV DNA ml$^{-1}$. Lanes 2–5 contain positive samples. (d) Extraction with the RTP DNA/RNA Virus Mini kit (Invitrogen) showing a sensitivity of $2 \times 10^7$ copies HBV DNA ml$^{-1}$. Lanes 2–7 contain positive samples. Lanes: 1: φX174, a molecular standard; 2–10, tenfold dilution series of HBV serum samples in oral fluid samples ($2 \times 10^6$ to $2 \times 10^{-2}$ copies HBV DNA ml$^{-1}$); 11, HBV serum as a positive control; 12: oral fluid as a negative control.

Methods for detection of HBV DNA in oral fluid samples

Four DNA extraction methods and three PCR protocols (an optimized semi-nested PCR, nested PCR and one-round PCR) were evaluated for detection of HBV DNA in oral fluid samples. Choosing the appropriate DNA extraction method is extremely important for obtaining valid and successful results in clinical samples. To our knowledge, there is no other extant study comparing DNA extraction methods for HBV detection in oral fluid samples. In the present study, each extraction method presented different recommended input and elution volumes, and three of the kits were designed for oral fluid samples, but the sample collection procedures recommended by the manufacturers were distinct from those employed in the present study. To avoid great differences in the standard protocol, and considering that saliva samples present lower DNA concentrations in relation to sera samples, we tried to increase the DNA quantity without great modifications to these protocols. For this reason, the elution volume was decreased for the High Pure PCR Template Preparation kit, the input volume was doubled for the QIAamp DNA Mini kit and water was not added to the swab when the RTP DNA/RNA Virus Mini kit was employed. Increasing sample volume has also been performed by Krause et al. (2006), who used oral samples for the detection of mumps and obtained good recovery.

The use of the QIAamp DNA Mini kit and the RTP DNA/RNA Virus Mini kit presented the highest sensitivities for nested PCR, detecting as few as 20 copies HBV DNA ml$^{-1}$. Both assays are based on silica adsorption, and the QIAamp DNA Mini kit has been used previously for the detection of the hepatitis A virus (HAV) and genomic DNA from oral fluid samples (Mackiewicz et al., 2004; Viltrop et al., 2010), and for fungal (Candida) (Metwally et al., 2008) and cytomegalovirus (CMV) DNA extraction (Evans et al., 1999) from blood samples. To our knowledge, there is no study in which the RTP DNA/RNA Virus Mini kit was used for HBV DNA detection in oral fluid samples.

One-round PCR with the RTP DNA/RNA Virus Mini kit gave the most satisfactory results among the oral fluid samples (20 copies ml$^{-1}$) because no smeared DNA was observed during the electrophoresis, demonstrating a good association between both methods. In the other methods, the smear bands were probably due to PCR inhibitory substances in the oral fluid samples, as no smear bands were observed when the same protocols were applied to serum samples (data not shown). The detection limit observed in the artificially spiked oral fluid samples in the present study was 20 copies HBV DNA ml$^{-1}$, which is lower than the value reported in the original study, 70 copies HBV DNA ml$^{-1}$, detected from serum samples using real-time PCR technology (Olioso et al., 2007). In contrast, one-round PCR with the Saliva DNA Isolation kit (Norgen) gave less satisfactory results ($2 \times 10^3$ copies HBV DNA ml$^{-1}$), which is likely to be because the collection method employed in the present study was distinct from that recommended by the supplier.

The sensitivities of the DNA extraction and detection methods are important, but other factors must also be considered when selecting the most practical and appropriate methodology. The cost of each method and the execution facilities required must also be taken into account.
the present study, the High Pure PCR Template Preparation kit (Roche) presented the lowest cost, whereas the Saliva DNA Isolation kit (Norgen) showed the highest cost. Conversely, the RTP DNA/RNA Virus (Invitek) is the most convenient method, as it includes a ready-to-use mixture of proteinase K, a nucleic acid carrier and an external extraction control. This mixture helps to reduce the time required to prepare this reagent and the contact with the infectious agent.

One limitation of this study was the impossibility of obtaining oral fluid samples from HBV-infected individuals; accordingly, only HBV-spiked samples were employed. However, the main objective of this study was to standardize the techniques for HBV DNA detection from oral fluid samples before sample collection is performed in clinical settings. Thus, the present study will give valuable information to future researchers in this area.

Another possible limitation of the present work is the absence of quantitative detection of HBV DNA. However, a qualitative assay was optimized for the detection of HBV DNA, which can be very useful for the identification of HBV occult infection, genotypes and mutants (Ocana et al., 2011; Said, 2011; Chakravarty, 2011; Guirgis et al., 2010; Valsamakis, 2007). For these reasons, the present study will be very useful for clinical studies regarding HBV diagnosis and the monitoring of HBV treatment.

In conclusion, all of the detection methods (semi-nested, nested and one-round PCR) can be used to detect HBV DNA from oral fluid samples, but the results obtained with one-round PCR and the RTP:\textsuperscript{®} DNA/RNA Virus Mini kit suggest that this combination is the best choice for HBV DNA detection. Oral fluid samples provide a non-invasive method of detecting HBV DNA that can be used for children and people with difficult venous access, such as the elderly, and the optimization of methods for HBV DNA detection is very important for improving the access to diagnosis in these populations.

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