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Importance of Collection Methods and Stability of Oral Fluid Samples for Hepatitis B Surface Antigen Detection

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Background: Oral fluid (OF) sample collection and stability for HBsAg detection are not fully established. This study aims to investigate the applicability of OF collectors and sample stability for Hepatitis B virus surface antigen detection. *Methods:* Paired serum and OF samples were obtained from 191 individuals, and Chembio (Chembio Diagnostic System, USA) and Salivette (Sarstedt, Germany) devices were used for OF collection. Two HBsAg enzyme immunoassays (EIAs) were used (HBsAg One kit, Radim, Rome, Italy and ETI-MAK-4, DiaSorin, Vercelli, Italy) to determine the most efficient method according OF collector. Sample volume, incubation time, and cutoff (CO) value were evaluated. The stability of OF samples was determined under different environmental conditions. *Results:* Chembio samples analyzed using DiaSorin EIA without modification of the manufacturer's instructions, demonstrated a sensitivity of 95.24% and a specificity of 100%. Salivette samples analyzed with Radim EIA with receiver operating characteristic (ROC) curve for calculating the CO showed a sensitivity of 78.26% and a specificity of 89.88%. HBsAg was detected in Chembio and Salivette samples under different environmental conditions, but the Chembio samples were the most stable. *Conclusions:* Both collectors can be used for HBsAg detection in OF samples, but some modifications of commercial EIAs should be incorporated for Salivette device. OF samples were reliably stable and could be stored for up to 90 days at 2–8°C. J. Clin. Lab. Anal. 27:186–194, 2013.

Key words: hepatitis B virus; oral fluid; diagnosis; stability study; collection device

INTRODUCTION

Hepatitis B virus (HBV) belongs to the Family *Hepadnaviridae* and the genus *Orthohepadnavirus*, and HBV infection is a global public health problem. It is currently estimated that more than 2 billion people worldwide have been infected, and approximately 360 million are living with HBV (1, 2). It is projected that 40% of these individuals will develop severe liver complications and are at risk of serious illness and death from liver cirrhosis and liver cancer (3).

The economic burden of HBV infection is substantial, and screening of infected individuals is important to identify the presence of chronically infected reservoirs, which can experience the serious and fatal complications of chronic liver disease and transmit the virus to other

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individuals, thereby increasing the incidence of the disease (4). In regions where financial resources are scarce, it would be beneficial to use methods with low cost and biological risk, such those that utilize oral fluid samples.

Traditionally, serum samples are used to detect antigens and antibodies against HBV, but the use of oral fluid samples would be appealing and advantageous due to their characteristics. Oral fluid collection is painless, cost-effective, simpler, and safer than blood collection. These advantages allow the collection of a large number of samples for epidemiological studies (5).

Several markers of viral infection have been detected in oral fluid samples, such as human immunodeficiency virus (6–8), Rubella virus (9), Epstein-Barr virus (10), hepatitis A virus (HAV) (11, 12), rotavirus (13, 14) poliovirus (15), and human T-lymphotropic virus (16). The presence of markers (HBsAg, anti-HBs, HBeAg, and anti-HBc) of HBV infection has also been demonstrated in oral fluid (5, 17–19), but different sensitivities and specificities were obtained depending on the oral fluid collector and the method of detection.

Therefore, it is important to evaluate oral fluid collection devices and detection methods for HBsAg to provide an accurate method for HBV diagnosis. Another important factor to be evaluated is the stability of oral fluid samples for HBsAg detection. Recently, Fung et al. (20) showed that HBsAg levels remained stable in stored frozen sera for 12 months without significant changes, but the stability of oral fluid samples for HBsAg detection has not been reported. Stability studies are important to evaluate the quality of results and to know if samples collected far from laboratory are able to be analyzed. In this respect, information on the stability of oral fluid samples is adequate to define the procedures for oral fluid collection and transportation, especially in difficult-to-access areas with poor sanitary conditions.

The main objective of this study was to evaluate different oral fluid sample collectors and to optimize HBsAg detection in oral fluid samples using commercial enzyme immunoassays (EIAs) to provide a more effective diagnosis. Therefore, to determine the most appropriate collection and storage methods, the stability of oral fluid samples for HBsAg detection was assessed using a panel of oral fluid samples obtained by two different collection methods and stored under different environmental conditions.

MATERIALS AND METHODS

Evaluation of Collector Performance and Optimization of HBsAg Detection Using Two Commercial EIAs

In order to evaluate the best association between the appropriate collection device and the commercial EIAs kits (ETI-MAK-4 [DiaSorin, Vercelli, Italy] and HBsAg One kit [Radim, Rome, Italy]), we used a compiling panel in which oral fluid samples were combined to make two pools of samples collected by each collector type. Oral fluid samples were obtained from 12 health professionals who kindly donated them for this study. After producing the pool, oral fluids were aliquoted into one of seven microtubes, and tenfold serial dilutions were performed to a final dilution of $1{:}10^6$ using a human plasma sample reactive for HBsAg with an optical density (OD) value of 3.0 by EIA and a viral load of 2,724.7 copies/ml. Estimated HBV concentration (in copies/ml) for each dilution was: 2.724 (dilution 10^{-1}), 272.4 (10^{-2}), 27.25 ($1{:}10^{-3}$), 2.72 ($1{:}10^{-4}$), 0.27 ($1{:}10^{-5}$), 0.03 ($1{:}10^{-6}$). A fraction of the oral fluid samples obtained from this pool was not spiked and was used as negative control for the experiment. HBsAg detection was performed on this serial dilution using two HBsAg EIAs, and the sample volume and sample incubation time were evaluated.

Oral fluid collected with the Chembio collector was tested using the ETI-MAK-4 commercial kit (DiaSorin) according to the manufacturer's instructions, and the sample volume was evaluated (100 μl as recommended by the manufacturer and a twofold increase to 200 μl). The same samples were also tested with the Radim EIA following the manufacturer's instructions, and the sample incubation time was evaluated (120 min at 37°C as recommended by the manufacturer and 960 min at 25°C). These modifications were performed to match the oral fluid OD to the paired serum OD.

The antigen concentration of oral fluid is much lower than that of sera (21). Consequently, the first incubation in the DiaSorin assay occurs without the addition of the conjugate, and the oral fluid volume was raised to increase the concentration of antigens reacting with the solid phase. In the Radim assay, the conjugate is added to the samples during the first incubation; because of this feature, the incubation time was increased to 18–24 hr as previously reported (5). These modifications were performed to improve the efficiency of the method for oral fluid samples.

Oral fluid samples obtained with the Salivette device were tested using the DiaSorin and Radim EIAs. The sample volume (for Diasorin assay, 100 μl as recommended by manufacturer and 200 μl [twofold increase]; for Radim assay, 150 μl as recommended by supplier) and the sample incubation time (60 min at 37°C, 120 min at 37°C and 960 min at 25°C) were evaluated.

Study Population

To evaluate the performance of the optimized EIA for oral fluid samples, paired serum and oral fluid samples were obtained during year of 2010 from two groups: (1) individuals living in remote areas in Pantanal in Mato

Grosso do Sul (Central Brazil) and (2) individuals referred to the Viral Hepatitis Laboratory (Oswaldo Cruz Institute) and the Clementino Fraga Filho University Hospital (UFRJ, Rio de Janeiro, Brazil).

The first group was composed of all individuals living in three remote communities of the Pantanal Region of Mato Grosso do Sul (Passo do Lontra, Serra do Amolar and Porto do Manga). The Passo do Lontra and Porto do Manga communities are located 300 and 385 km from Campo Grande City (capital of Mato Grosso do Sul state), respectively, whereas Serra do Amolar is located 217 km (waterway) from Corumbá (municipality of Mato Grosso do Sul state). These communities were chosen due to the difficulty in accessing this region. All individuals at least 18 years of age that agreed to participate in this study were included. The individuals of this group without HBV infection were considered healthy. However, in the present study, blood samples were obtained and tested for HBV markers to prove this hypothesis.

The second group was composed of suspected and/or confirmed cases of HBV infection that were referred to two Viral Hepatitis Centers in Rio de Janeiro. These centers were responsible for diagnosing most of the HBV cases in the state because they attend approximately 800 individuals per month.

In the first group, 144 volunteers provided paired serum and oral fluid samples, which were obtained with the Chembio and Salivette commercial collectors. In the second group, 47 individuals provided paired serum and oral fluid samples with the two devices.

The Ethics Committee of the Oswaldo Cruz Institute approved this study (number 433/07), and it was conducted in accordance with the ethical principles of the Helsinki declaration. Written informed consent was obtained from each individual prior to enrolment in the study.

Biological Samples

Blood samples were collected from all volunteers by venepuncture using sterile vacutainer tubes and were processed to obtain serum samples. Oral fluid samples were obtained using two commercial collectors: Chembio and Salivette. The Chembio collector consists of a handle with a sponge at the end, which was rubbed between the teeth and gums for approximately 1 min; after the swab was placed in a plastic bottle containing 500 μl of a specific buffer. Then, the oral fluid was concentrated at the bottom of the plastic tube by centrifugation at 1,400 × *g* at 25°C for 10 min and stored at 2–8°C.

The Salivette collector consists of a cylindrical cotton sponge that is placed between the gum and cheek at the bottom of the mouth for 2 min after 1 ml of Phosphate buffer saline (PBS)/0.5% Bovine serum albumine (BSA) transport buffer was added to the device, and the sample was centrifuged for 1,400 × *g* for 10 min (5).

All oral fluid and serum samples were collected at the same time and Salivette and serum samples were stored at −20°C instead of Chembio samples that were stored at 2–8°C after processing until analyses.

Enzyme Immunoassays

HBsAg was detected in serum and oral fluid samples using commercial EIAs. Serum samples were tested using two different assays according to the manufacturer's instructions (ETI-MAK-4 [DiaSorin] and HBsAg One kit [Radim]). Initially, oral fluid samples obtained with both collectors were tested as described. After optimization, each sample was tested using the best EIA; however, Salivette samples were tested using the HBsAg One kit as described by the manufacturer and optimized as described previously (5). Cutoff (CO) values were also evaluated for the HBsAg One kit, for which a receiver operating characteristic (ROC) curve was employed.

Both EIAs (ETI-MAK-4 and HBsAg One) are direct, noncompetitive tests based on the use of polystyrene microwells coated with mouse monoclonal antibodies against HBsAg (directed against the "a" determinant of HBsAg). An enzyme tracer containing horseradish peroxidase-labeled sheep antibodies against HBsAg detects the captured HBsAg from a patient's specimen.

In the ETI-MAK-4 EIA, patient's specimens (serum or oral fluid) and controls are deposited on a plate with solid phase composed of anti-HBs along to incubation buffer without enzyme tracer, whereas for the HBsAg One kit, the sample and the enzyme tracer are incubated in this first step. If HBsAg is present in a specimen or control, it binds to the antibodies. Excess sample is removed by a wash step, and the enzyme tracer is then added to the microwells (for ETI-MAK-4) and allowed to incubate. The enzyme tracer binds to any antigen–antibody complexes present in the microwells. Excess enzyme tracer is removed by a wash step, and a chromogen/substrate solution is added to the microwells and allowed to incubate. If a sample contains HBsAg, the bound enzyme (horseradish peroxidase) chemically reduces the substrate peroxide, which concurrently oxidizes the chromogen tetramethylbenzidine (TMB) to a blue color (650 nm). The blue color turns to yellow (450 nm) after the addition of the stop solution. If a sample does not contain HBsAg, the microwell will be colorless after the chromogen/substrate solution is added and will remain colorless after the stop solution is added. Color intensity, which is measured spectrophotometrically, is indicative of the concentration of HBsAg. Absorbance value readings for patient specimens are compared with a CO value.

The CO values for HBsAg detection were evaluated using three calculation methods. In the first method, EIA ETI-MAK-4 (Diasorin) instructions were followed when samples were tested by this method (0.03 + CNX, where CNX is the average of the negative controls from the assay). In the second CO method, HBsAg One (Radim) instructions were followed when samples were tested by this method (0.05 + CNX, where CNX is the average of the negative controls). Samples were classified according OD/CO criteria; positive samples presented OD/CO values equal to or higher than 1.1, negative samples presented OD/CO values lower than 1.0, and undetermined samples showed OD/CO values between 1.0 and 1.1. In the third CO calculation formula, results from sera samples were compared to OD values of oral fluid samples using ROC curve analysis. ROC curve analysis establishes the CO value that offers the best values of sensitivity and specificity.

To describe HBV serological status of each individual, serum samples were also tested for the presence of anti-HBc (ETI-AB-COREK PLUS, DiaSorin) and anti-HBs (ETI-AB-AUK-3, DiaSorin) using commercial EIAs according to the manufacturer's instructions.

Evaluation of the Stability of HBsAg in Oral Fluid Samples

To evaluate the stability of oral fluid samples for HBsAg detection, oral fluid samples were collected with Chembio or Salivette from 11 individuals (health workers) without HBV markers (HBsAg, anti-HBc, and anti-HBs). These samples were mixed, and a portion was used as negative control, whereas the other portion was spiked with a positive HBsAg serum sample (OD/CO value greater than 3.0 in both EIAs and a viral load of 2,724.7 copies of HBV DNA/ml) to produce a sample containing 2.72 copies of HBV DNA/ml. This sample, negative oral fluid samples, and serum controls (reactive and nonreactive for HBsAg) were stored under the following conditions: 2–8°C for 90 days; 20–25°C for 15 or 30 days; 37°C for 7 days (equivalent to 1 year at 4°C), 15 days (equivalent to 2 years at 4°C), or 30 days (equivalent to 4 years at 4°C); 50°C for 30 days (equivalent to 1 year at 25°C) or 60 days (equivalent to 2 years at 25°C); or −20°C for 60 days.

The storage conditions were based on the environmental conditions used for accelerated stability studies (22), in which specific conditions mimic the storage of samples for a longer period of time. This evaluation was performed to determine the time and temperature necessary for oral fluid collection and transportation to the laboratory and to determine the storage conditions of these samples in the laboratory.

Statistical Analysis

The data obtained were coded and entered into a database created in Excel (Microsoft Office). HBsAg detection in serum samples was used as the gold standard for the assessment of sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) of each collector. HBsAg serum gold standard assay was HBsAg One (Radim) for oral fluid samples collected using Salivette and ETI-MAK-4 (Diasorin) for samples collected using Chembio. Descriptive statistical analysis was performed to calculate frequencies, mean ± standard deviation (SD), median, and confidence intervals (CI). To evaluate the deviation between the observed and expected values in the groups, χ^2 test or Fisher's exact test were used. The Kappa coefficient (k) was used to assess the degree of agreement between oral fluid and serum antibody status according to the following interpretation: $k < 20\%$, poor; $k = 21$–40%, fair; $k = 41$–60%, moderate; $k = 61$–80%, good; and $k = 81$–100%, excellent agreement (23). Two-tailed P values <0.05 were considered statistically significant. All calculations were performed in GraphPad InStat ® 3.01 (GraphPad Software, San Diego, CA).

RESULTS

Establishment of HBsAg Detection in Oral Fluid Samples Obtained With Two Different Devices

Oral fluid collected with a Chembio collector demonstrated the highest sample OD/CO ratio (8.51) using the ETI-MAK-4 kit with a twofold increase in sample volume, but the limit of detection was lower than with the volume recommended by the manufacturer (100 μl). Using these samples and the Radim EIA, the best results were also obtained using the protocol recommended by the manufacturer, with a limit of detection of $1{:}10^3$ (Table 1).

Oral fluid samples collected with a Salivette collector and tested with the ETI-MAK-4 assay exhibited the highest performance with a twofold increase in sample volume and an extended sample incubation time (from 60 to 960 min) (OD/CO = 6.7). HBsAg was detected in these samples with the HBsAg One assay to a dilution factor of $1{:}10^6$ with the sample volume recommended by the manufacturer and an extended sample incubation time (from 60 to 960 min), but low variation was observed among the different protocols (Table 1).

In light of these results, the Chembio samples were tested using the ETI-MAK-4 assay as recommended by the manufacturer and Salivette samples were tested using the HBsAg One assay as described by the supplier (150 μl of sample and 120 min of sample incubation time). For stability studies, Salivette samples were tested as described

TABLE 1. HBsAg Detection Limit in Oral Fluid Samples Obtained with Two Commercial Devices (Salivette and Chembio)

EIA manufacturer	Oral fluid volume (μl)	Incubation time (min)[a]	Mean OD/CO[b]	Value in nm (dilution factor)
			Chembio	Salivette
ETI-MAK-4 DiaSorin	100	60	1.54 ($1:10^5$)	1.70 ($1:10^5$)
	100	960	ND	5.2 ($1:10^5$)
	200	60	8.51($1:10^4$)	1.19 ($1:10^5$)
	200	960	ND	6.7 ($1:10^5$)
HBsAg One Radim	150	120	1.66 ($1:10^3$)	1.99 ($1:10^5$)
	150	960	6.63 ($1:10^1$)	2.21 ($1:10^6$)

The detection limits were obtained using a tenfold serially spiked with an HBV serum sample (OD/CO value greater than 3.0 by EIA and a viral load of 2,724.7 copies/ml).
[a]The standard protocol for the DiaSorin assay employs 100 μl of sample and 60 min of sample incubation, whereas for Radim, 150 μl of sample and 120 min of sample incubation are needed.
[b]Ratio of the sample OD and the CO value.
ND, not done.

by Cruz et al. (5) with an increase in sample incubation time from 120 to 960 min.

Demographic Data

In this study, paired oral fluid and serum samples were collected from two different groups, and two distinct commercial oral fluid collectors were used (Chembio and Salivette). The first group consisted of 144 volunteers from a riverside area of Pantanal of Mato Grosso do Sul (Central Brazil), and the second group consisted of 47 volunteers from Viral Hepatitis Centers (Rio de Janeiro, Brazil) who were confirmed or suspected cases of HBV infection. In the first group, 54.2% (78/144) were male and 45.8% (66/144) were female. The age ranged from 3 to 77 years, with a mean (±SD) of 29.4 years (±19.27). In the second group, there was a predominance of women (57.5%), and all were 15–75 years old with a mean (±SD) age of 47.6 years (±15.5).

HBV Markers in Serum Samples

In this study, the overall prevalence of HBsAg was 10.9% (21/191). Serum samples were tested for the presence of HBsAg using the DiaSorin and Radim EIA kits, and two discordant results were obtained. Using the HBsAg One kit, HBsAg was detected in 23 samples (12.0%), whereas HBsAg was present in 21 samples (10.9%) using the ETI-MAK-4 EIA. These discordant samples did not exhibit anti-HBc or anti-HBs. These samples were not excluded from the analysis, since each assay was employed as gold standard method among sera samples in comparison to oral fluid samples obtained using Salivette or Chembio device.

Among the study participants, 9.4% (18/191) had seroconverted to anti-HBs after being previously infected (anti-HBc total/anti-HBs), 2.1% (4/191) were infected (isolated total anti-HBc), and 36.6% (70/191) exhibited no HBV markers. Anti-HBs was detected as isolated marker from 27.7% (53/191) of participants.

TABLE 2. Hepatitis B Virus Serological Markers in Serum Samples in the Studied Population

HBV marker	Group 1	Group 2	Total
	Pantanal-MS (%) $n = 144$	VHC (%) $n = 47$	(%) $n = 191$
HBsAg			
Positive	1 (0.7)	20 (42.5)[a]	21 (10.9)
Negative	143 (99.3)	27 (57.4)	170 (89.0)
Anti-HBc/anti-HBs	14 (9.7)	4 (8.5)	18 (9.4)
Anti-HBc	2 (1.4)	2 (4.2)	4 (2.1)
No markers	60 (41.7)	10 (21.3)	70 (36.6)
Anti-HBs	43 (29.9)	10 (21.3)	53 (27.7)

[a]Sera tested with the Radim kit showed 22 HBsAg reagent (22/47).
VHC, Viral Hepatitis Centers.

The analysis of HBV markers in serum samples revealed one HBsAg-positive individual (0.7%), 14 individuals that were previously infected with HBV (9.7%), 43 (29.9%) individuals presenting anti-HBs, and 60 (41.7%) individuals susceptible to HBV infection (presenting no HBV markers) in the first group (individuals from Pantanal). Among the second group (Viral Hepatitis Centers), 42.5% (20/47) were positive for HBsAg, 8.5% (4/47) had previous contact with HBV, 21.3% (10/47) were anti-HBs-positive, and 21.3% (10/47) presented no HBV markers (Table 2).

Detection of HBsAg in Oral Fluid Samples Collected Using Different Collectors

Oral fluid samples were tested as described above. Comparing the entire population (191 paired samples), the Chembio collector and ETI-MAK-4 EIA showed high

TABLE 3. Performance of the HBsAg Test (ETI-MAK-4) of Oral Fluid Collected with the Chembio Device According to the Population Studied

Population	*N*	Sensitivity% (CI 95%)	Specificity% (CI 95%)	PPV% (CI 95%)	NPV% (CI 95%)	*k* (%)
Entire population	191	95.2 (76.2–99.9)	100 (97.85–100)	100 (83.16–100)	99.42 (96.78–99.99)	97
General population Pantanal (MS)	144	100 (2.5–100.0)	100 (97–100)	100 (2.5–100)	100 (97.45–100)	100
Viral Hepatitis Centers Rio de Janeiro (RJ)	47	95 (73.15–99.87)	100 (87.23–100)	100 (82.35 – 100)	96.43 (81.65–99.91)	95

PPV, positive predictive value; NPV, negative predictive value; CI, confidence interval.

TABLE 4. Accuracy Indices for HBsAg Detection in Oral Fluid Samples Collected using the Salivette Device and Tested with HBsAg One According to the CO Value

	Manufacturer's CO: NCX + 0.05		ROC analysis curve 0.024	
	$n = 190$[a]	(CI 95%)	$n = 191$	(CI 95%)
Sensitivity%	31.8	(13.9–54.9)	78.3	(53.3–92.5)
Specificity%	99.4	(96.7–99.9)	89.9	(84.3–93.9)
PPV%	87.5	(47.3–99.7)	51.4	(99.9–68.7)
NPV%	91.8	(86.8–95.3)	96.8	(92.7–98.9)
k%	43		55	

[a]Total does not equal 191 individuals because one pair of serum and oral fluid samples gave an undetermined result.
NCX, negative control media; ROC, receiver operating characteristic; PPV, positive predictive value; NPV, negative predictive value; CI, confidence interval.

levels of agreement (97%), sensitivity (95.2%), and specificity (100%). When these parameters were evaluated within each group, the sensitivity was higher in the general population (100%) than among suspected cases of HBV infection (95%; Table 3).

Salivette samples tested with the HBsAg One EIA using a CO value according to the supplier's instructions showed low sensitivity (31.8%) compared with ROC curve analysis for CO calculation. When samples with OD values greater than 0.024 were considered positive, sensitivity increased more than twofold (78.2%), whereas specificity and PPV decreased (89.8% and 51.4%, respectively), and kappa values were similar for both methods (43% vs. 55%; Table 4). Using this optimized assay, high sensitivity and specificity were observed for the general population (100% and 94.4%, respectively) compared with the suspected HBV cases (77.3% and 64.0%; Table 5).

Evaluation of HBsAg Stability in Oral Fluid Samples Obtained Using Two Different Collectors

To evaluate the stability of HBsAg, serial dilutions of HBsAg-reactive sera in oral fluid samples were tested after storage under different conditions. No false positive or negative results were obtained using HBsAg-spiked or HBsAg-negative oral fluid samples.

Chembio and Salivette samples were highly stable at 2–8°C for 90 days and at −20°C for 60 days. At room temperature (22–25°C), both samples were also stable, but no variation in OD/CO values was observed with Salivette samples.

At 37°C, HBsAg could be detected in Chembio and Salivette samples for up to 30 days, which is equivalent to storage for 4 years at 4°C, but the OD/CO values tended to decrease. At 50°C, HBsAg could be detected for 60 days in both samples, which is equivalent to 2 years at 25°C; however, the OD/CO values tended to decrease for Chembio samples and increase for Salivette samples (Table 6).

DISCUSSION

HBV diagnosis is usually made using serum samples that requires venepuncture, specialized technicians, and biosafety conditions. Oral fluid samples can be an alternative for HBV diagnosis, thereby improving access for several groups, such as children and the elderly. In the present study, HBV surface antigen was detected in oral fluid samples obtained using two different collectors; however, some modifications should be performed to adapt commercial EIAs for HBsAg detection. Moreover, the stability of oral fluid samples for HBsAg detection was also determined.

Two oral fluid collectors were evaluated (Chembio and Salivette devices), and the Chembio device combined with the ETI-MAK-4 EIA gave highest sensitivity and specificity. One possible reason for this difference can be the type of collection because the Chembio device employs a mechanical mechanism, whereas the Salivette device employs an adsorption mechanism. Mechanical collection allows for the acquisition of gingival crevicular fluid and for increased vascular permeability, leading to an accumulation of fluid with a small amount of food debris and a greater concentration of HBsAg in the sulcus (12,24). Another factor may be the type of elution buffer used in each

TABLE 5. Performance of the HBsAg Assay on Oral Fluid Samples Collected using Salivette Devices and Tested with HBsAg One According to the Population Studied; CO Values Based on ROC Curve Analysis

Population	*n*	Sensitivity% (CI 95%)	Specificity% (CI 95%)	PPV% (CI 95%)	NPV% (CI 95%)	*k* (%)
Entire population	191	78.3 (56.3–92.5)	89.9 (84.3–93.9)	51.4 (33.9–68.6)	96.8 (92.7–98.9)	55.0
General population Pantanal (MS)	144	100.0 (2.5–100.0)	94.4 (89.3–97.5)	11.1 (0.2–48.2)	100.0 (97.3–100.0)	18.0
Viral Hepatitis Centers Rio de Janeiro (RJ)	47	77.3 (54.6–92.2)	64.0 (42.5–82.0)	65.4 (44.3–82.8)	76.19 (52.8–91.8)	40.0

PPV, positive predictive value; NPV, negative predictive value; CI, confidence interval.

collector because the Chembio device employs a buffer developed by the manufacturer that contains substances that increase the sensitivity of the method, whereas the 1 ml of transport buffer used with the Salivette dilutes these samples, and consequently, the necessary amount of analyte cannot be recovered from the collector's fibers (21). Tourinho et al. (12) have employed Chembio® device and an "in-house" competitive EIA for detection of total anti-HAV antibodies (IgG and IgM) and showed an excellent performance of this device to obtain HAV antibodies among oral fluid samples. However, at our knowledge this is the first time that this device has been employed for HBsAg detection. Salivette device has been used previously for HBsAg detection (5) showing higher sensitivity and specificity compared to the present study probably due to the high HBV prevalence in earlier study (5). In the present study, optimized assay using Salivette device present high sensitivity in low endemicity regions, but the interval of confidence was relatively large and low positive predictive value was observed. On the other hand, in the group of individuals suspected or with HBV infection, positive predictive values of this assay were relatively high compared to low endemicity area, since it is easier to find true positives in high endemicity regions.

In the present study, it was also observed that different commercial EIAs were appropriate for HBsAg detection when the two different oral fluid devices were employed. For Salivette samples, HBsAg One provided good results when the incubation time was increased, as shown by Cruz et al. (5); however, in the present work, this modification was not performed because the OD values obtained with the protocol provided by the manufacturer also offered satisfactory results. It is likely that the Salivette samples gave a low HBsAg concentration and because HBsAg One EIA employs the simultaneous addition of sample and conjugate, this can improve the sensitivity of the method. Samples with low concentrations (oral fluid samples) of HBsAg may require more time for specific attachment to the solid phase of the assay, and after this process, HBsAg can bind to the conjugate, thereby reducing nonspecific reactions.

The sample volume was also an important factor in the EIA assays; however, there was no need to increase the sample volume using Chembio or Salivette samples in both assays as other authors have described to obtain better results using oral fluid samples (5,11,25,26). However, for Salivette devices, the CO should be changed to increase the sensitivity as has been previously shown for the

TABLE 6. OD/CO Values (nm) of Oral Fluid Samples Obtained using Chembio and Salivette Devices According to Different Storage Temperatures and Periods of Time

Environmental condition (temperature in degrees Celsius)	Period of time (days)									
	Chembio oral fluid device and ETI-MAK-4 EIA					Salivette oral fluid device and HBsAg One kit				
	7	15	30	60	90	7	15	30	60	90
−20°C	NT	NT	NT	81.08	NT	NT	NT	NT	63.83	NT
2–8°C	NT	NT	NT	NT	77.73	NT	NT	NT	NT	63.83
25°C	NT	45.8	56.49	NT	NT	NT	63.83	63.83	NT	NT
37°C	59.89	43.03	26.53	NT	NT	63.83	63.83	36.52	NT	NT
50°C	NT	NT	21.64	1.27	NT	NT	NT	9.98	63.83	NT

The CO value for the Chembio Device was determined using the ETI-MAK-4 EIA and by Cruz et al. (16) for the Salivette Device.
NT, not tested.

detection of other hepatitis viruses in oral fluid samples (5, 12, 27).

Using the Chembio samples, no changes should be performed with commercial EIAs developed for serum samples, which indicates that this method can be employed with high sensitivity and specificity for HBsAg detection in oral fluid samples. Previous studies have attempted to use EIAs developed for serum samples with oral fluid samples, but this has resulted in nonspecific results (17, 18). However, Salivette samples in combination with the Radim EIA requires ROC curve analysis for CO calculation to improve the sensitivity of the method, which confirms the importance of the association between the collection and detection methods. Using Salivette samples and HBsAg One (Radim) assay, the CO value was too low to increase the false positive results, thus Chembio device along with Diasorin assay are more appropriate for HBsAg detection among saliva samples. However, sample collection using Salivette is easy and cheap allowing HBV detection in resource limited areas. Further investigations are being conducted in order to evaluate the feasibility of these samples for HBV epidemiological studies.

This present study has some limitations, for example, the small number of HBV-infected individuals that could yield high predictive values, the absence of immunocompromised individuals that can interfere with sensitivity, and finally, the absence of HBsAg quantification in saliva and serum samples to evaluate the correlation between HBsAg levels in both samples.

HBV DNA levels (28) and the stability of HBsAg (20) have been demonstrated in serum samples, but to our knowledge, HBsAg stability has not been established in oral fluid samples. In this study, the Chembio samples were highly stable, although both samples could be used for HBsAg detection under all of the environmental conditions tested. These data demonstrate that storage at 2–8°C for 90 days is the best option for Chembio samples. In accelerated stability studies, HBsAg could be detected for up to 4 years at 4°C and 2 years at 25°C in both samples.

In the present study, OD/CO values varied according to temperature and length of storage, and the lowest values were obtained at 50°C for 60 days (corresponding to 2 years at 25°C) for the Chembio samples and at 37°C for 30 days (corresponding to 4 years at 4°C) for both the Chembio and Salivette samples. OD/CO values tend to decrease for Chembio samples and increase for Salivette samples, probably due to the low specificity of HBsAg assay using Salivette devices and high sensitivity of HBsAg assay using Chembio devices. These data are supported by high OD/CO values of Salivette samples stored for 60 days at 50°C in comparison to 30 days showing that high unspecific results are observed for long period of storage using Salivette samples.

Previous studies have shown that HBV DNA and HCV RNA load can also increase with progressive freeze-thaw cycles (28, 29), whereas intact HCV RNA cannot be detected in samples collected with a Salivette over a period of 1 month at −20°C (30), which emphasizes the impact of the selection of the oral fluid collection device and its association with HBsAg detection.

In conclusion, HBsAg could be detected in oral fluid collected with Chembio and Salivette collectors, but different commercial EIAs are necessary. However, the Chembio device in combination with the ETI-MAK-4 EIA, which gave the highest sensitivity and specificity, is better suited for HBsAg detection. The Chembio and Salivette samples were relatively stable for HBsAg detection under different environmental conditions, and the lowest variation in OD/CO values was observed for 90 days at 2–8°C and 60 days at −20°C. These data show that oral fluid samples are promising biological samples for HBsAg detection within different HBV-endemic populations and are a good choice for hepatitis B prevalence studies in resource-limited areas where cold storage transportation and processing conditions are not available.

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The authors declare that they have no conflict of interest.

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