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Evaluation of Saliva Specimens as an Alternative Sampling Method to Detect Hepatitis B Surface Antigen

Helena Medina Cruz,[1] Elisangela Ferreira da Silva,[1] Cristiane A. Villela-Nogueira,[2] Letícia C. Nabuco,[2] Kycia Maria Rodrigues do Ó,[2] Lia Laura Lewis-Ximenez,[1] Clara Fumiko Tachibana Yoshida,[1] Elisabeth Lampe,[1] and Livia Melo Villar[1]*

[1]*Laboratory of Viral Hepatitis, Oswaldo Cruz Institute, FIOCRUZ, Rio de Janeiro, Brazil*

[2]*Hepatology Division, Clementino Fraga Filho University Hospital, Federal University of Rio de Janeiro, Rio de Janeiro, Brazil*

In this study, a modified enzyme immunoassay (EIA) was evaluated for the Hepatitis B surface antigen (HBsAg) among whole saliva and oral fluid samples. Specimens were collected from 115 individuals who gave serum and oral fluid using Salivette (Sarstedt, Nümbrecht, Germany) and whole saliva. Saliva specimens were tested following a modified ELISA, and the results were compared with paired serum specimens that were tested according to the supplier's instructions. Transport buffer for the oral fluids, sample volume for assay, incubation period of sample with conjugate as well as cut-off values were evaluated to optimize the assay. The highest sensitivity and specificity were obtained by increasing the incubation of sample and conjugate to 16 hr and using the area under the receiver operating characteristic curve to calculate cut-off values. HBsAg was detected in 40 oral fluids and 44 whole saliva samples out of 47 paired positive serum specimens and not detected in 64 oral fluids and 63 whole saliva samples out of 68 matched negative sera samples by the ELISA assay. There was excellent agreement between the results for the serum and saliva specimens kappa value (κ): 0.80 for oral fluid and κ: 0.87 for whole saliva and there was excellent reproducibility. Using an optimized protocol, the sensitivities of whole saliva and oral fluid were 93.6 and 85.1%, respectively, whereas specificities of whole saliva and oral fluid were 92.6 and 94.1%, respectively. Our data showed a significant promise for the use of whole saliva and oral fluid together with the modified commercial EIA for Hepatitis B virus infection surveillance. J. Clin. Lab. Anal. 25:134–141, 2011. © 2011 Wiley-Liss, Inc.

Key words: Hepatitis B; whole saliva; oral fluid; enzyme immunoassay

INTRODUCTION

Hepatitis B virus (HBV) infection is a major cause of chronic hepatitis worldwide and HBsAg testing is a global issue, as a WHO program requests HBV vaccination for newborns of HBV-infected mothers. It has been established that HBV transmission is hematogenic, sexual, and perinatal, but transmission by saliva has been demonstrated in experimental studies with gibbons (1,2). The presence of HBV DNA in saliva has been described showing the potential infectivity of this specimen (3–6).

Traditionally, HBV is diagnosed through HBV antigen or antibody detection in serum or plasma derived from whole blood collected by venipuncture and hence requires trained health-care workers for collection and laboratory facilities for testing. Over the past years, alternative fluids for viral diagnosis, such as saliva, urine, and dried blood spots, have been widely studied (7–12).

Saliva can be considered as whole saliva or oral fluid. Whole saliva is a mixture of oral fluids and includes secretions from both the major and minor salivary glands, in addition to several constituents of nonsalivary origin, such as gingival crevicular fluid (GCF), bronchial

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*Correspondence to: Livia Melo Villar, Laboratório de Hepatites Virais Pavilhão Helio e Peggy Pereira-Térreo-sala B09, Instituto Oswaldo Cruz, FIOCRUZ. Av. Brazil, 4365 Manguinhos-RJ. Cep:21045900. Rio de Janeiro, Rio de Janeiro, Brazil. E-mail: lvillar@ioc.fiocruz.br

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expectorated and nasal secretions, serum and blood derivatives from oral wounds, bacteria and bacterial products, viruses and fungi, desquamated epithelial cells, other cellular components, and food debris (13). Unstimulated whole saliva is collected without exogenous gustatory, masticatory, or mechanical stimulation asking the subject to spit whole saliva into a test tube. Oral fluid contains principally GCF that is an ultra-filtrate of plasma that enters the oral cavity by transudation from capillaries present in the mucosa of the gingival space. Oral fluid is obtained using collection devices such as Salivette, Oracol, Orasure, Omnisal, and it can be used to detect viral hepatitis markers (9,12,14,15). Oral fluid can be collected by placing the tip of a collection device at the orifice of the Wharton's duct, after placing sterile cotton sponges in the floor of the mouth and over the buccal mucosal areas to occlude the parotid and sublingual ducts (13).

Collection of whole saliva or oral fluid samples is less expensive, less invasive, and less painful compared with blood collection. Using whole saliva or oral fluid samples as alternative fluids for HBV testing could be a useful tool for epidemiological purposes, especially when blood collection is difficult (small children, intravenous drug users, or hemophiliacs) or for field collection of samples in remote areas or in nonclinical settings by persons with minimal training.

The sensitivity and specificity of assays designed to detect HBV markers in serum samples can differ when these tests are employed for detection in whole saliva or oral fluid specimens (3,11,16–21). The inconvenience of saliva samples is the fact that the concentration of viral markers present in this fluid is lower in comparison with plasma (22). So, the type of saliva collection and assay used to detect viral markers should be evaluated to obtain an accurate diagnosis. This study was designed to evaluate the use of a modified ELISA for the Hepatitis B surface antigen (HBsAg) among whole saliva and oral fluid samples and to show different methods to obtain saliva for HBV detection, principally in developing areas with limited resources to buy collection devices to obtain such specimens.

MATERIALS AND METHODS

Study Population

Paired serum, whole saliva, and oral fluid samples were obtained from HBV-infected patients visiting the Viral Hepatitis Laboratory, FIOCRUZ and Hospital Universitario Clementino Fraga Filho, Federal University of Rio de Janeiro. HBV-negative individuals were also included as controls. Data concerning HIV status and the severity of HBV disease were unknown. All study participants gave informed consent. Ethical approval for the study was obtained from the Oswaldo Cruz Foundation and Hospital Universitário Clementino Fraga Filho Ethics Committee.

The sample set included 47 HBsAg serum-positive samples and a control group of 68 HBsAg serum-negative samples. Mean age of the population studied was 44 years old (SD: 14, range: 15–80 years) and 51% were female.

Sample Collection and Processing

Blood samples were obtained by venipuncture with Vacutainer tubes, after which serum was obtained by centrifugation and stored at -20°C until testing (12). Oral fluid was obtained using a commercial device (Salivette, Sarstedt) where 500 ± 800 ml of oral fluid was collected and drowned with 1 ml of transport buffer. Oral fluid samples were centrifuged ($1{,}400 \times g$ for 10 min) and stored at -20°C until assayed. Whole saliva samples were obtained without previous stimulation by asking the patient to spit out into a sterile container. Whole saliva specimens were obtained shortly after oral fluid collection until the participant could produce saliva again. The samples were stored in microtubes at -20°C until testing. The samples (blood, whole saliva, and oral fluid) were collected subsequently on the same day. Whole saliva and oral fluid were macroscopically seen to contain blood as previously described (10). When contamination occurred, the samples were excluded. In this study, no samples presented any blood contamination.

Enzyme Immunoassay

All samples were analyzed by the manual technique (HBsAg One, RADIM, Pomezia (Roma), Italy) designed to detect HBV surface antigen in serum. Serum specimens were tested according to the manufacturer's instructions. This test is based on an enzyme immunoassay (EIA) where two different anti-HBsAg monoclonal antibodies are used, one adsorbed on the wells and the other conjugated to horseradish peroxidase. During the first incubation (120 min at 37°C), 150 µl of the sample was incubated and if HBsAg was present in the sample, it would bind to both monoclonals at once, by forming a "sandwich." Following this incubation, the unbound material is removed by an aspiration and washing cycle. The residual enzyme activity found in the wells will thus be directly proportional to HBsAg concentration in the samples and evidenced by incubating the solid phase with 100 µl of Chromogen solution (Tetramethylbenzidine) in a substrate-buffer. Colorimetric reading was performed by using a spectrophotometer at a 450 nm wavelength (reference filter 620 nm).

Quality Parameters

For whole saliva and oral fluid specimens, feasibility studies were carried out using a panel of ten paired

serum, whole saliva, and oral fluid samples obtained from five HBV-infected individuals and five healthy individuals (23). In feasibility studies, the parameters evaluated were: (a) transport buffer for oral fluid samples [(i) phosphate buffer saline (PBS) pH 7.2; (ii) PBS/Tween 20 0.05%; (iii) PBS/Tween 20 0.05%/ 0.005% sodium azide; (iv) PBS/Tween20 0.2%/ bovine serum albumin (BSA) 5%, and (v) PBS/BSA 0.5%]; (b) type of whole saliva (whole saliva, pellet and supernatant, the latter was obtained by centrifuging whole saliva at 1,600 rpm, for 20 min at 4°C (7) and pellet was resuspended with 1 ml of distilled water until testing); (c) volume of sample input for whole saliva and oral fluid (150 µl; 200 µl, and 250 µl), and (d) time of incubation of whole saliva or oral fluid sample with commercial conjugate provided by the EIA manufacturer (RADIM) (90 min at 37°C and 18 ± 2 hr at room temperature).

All transport buffers were produced by the authors at the Viral Hepatitis Laboratory using commercial reagents. For PBS $1\times$, NaCl (Sigma-Aldrich, St. Louis, MO), KCl (Sigma), Na_2HPO_4, and KH_2PO_4 (Merck, Darmstadt, Germany) and distilled H_2O were used (24). Tween 20 or polysorbate 20 (Sigma) can be used to remove unbound immunological coumpounds, and eventually for incubation solutions of immuno-reagents (labeled antibodies) to reduce an unspecific background. Sodium azide (Merck) is a common preservative of samples and there are stock solutions in laboratories. BSA cat no. A7906 (Sigma) is a commonly used ELISA blocking agent (24).

As whole saliva or oral fluid samples are not routinely used to screen patients, there are no standard guidelines to calculate the cut-off absorbance value for these specimens. Therefore the absorbance value for whole saliva or oral fluid samples above which samples were considered positive, was calculated by three methods: In the first method, the manufacturer's recommendation to calculate the cut-off was used (CO1). In the second method, three standard deviations above the mean whole saliva or oral fluid absorbance of HBV seronegative samples (mean+3SD) were chosen as cut-off (CO2), which included all negative samples values. In the third method, the area under the receiver operating characteristic curve (AUROC) (23,25) was performed for whole saliva and oral fluid absorbance values using the MedCalc statistical software (version 9.2.1.0, MedCalc Software, Mariakerke, Belgium) (CO3).

Using the optimized assay, reproducibility was evaluated by spiking HBsAg reactive serum samples among whole saliva and oral fluid samples obtained from healthy donors. Healthy donors did not present HBsAg, anti-HBc, or anti-HBs markers in their serum samples. HBsAg reactive serum is an internal quality control developed at the Viral Hepatitis Laboratory with an OD value above 3.0. Serial ten-fold dilution was prepared to determine end point dilution and reproducibility of the test. Each dilution was analyzed in duplicate on three different days.

Data Analysis

HBsAg detection in serum samples was used as the gold standard for the assessment of sensitivity, specificity, positive (PPV), and negative (NPV) predictive values of whole saliva and oral fluid HBV assay. Descriptive statistics are shown as the mean $\pm$ standard deviation or the median and interquartile range as appropriate. Categorical variables were compared between groups by the χ^2 test or Fisher's exact test, and noncategorical variables were compared by the Mann–Whitney U test. A P value of <0.05 was considered significant. Concordance between results obtained for whole saliva or oral fluid samples and matched serum samples was established using the Kappa index. All data analysis was performed using the program 3.01 (GraphPad InStat®software, San Diego, CA).

RESULTS

The ability to detect HBsAg in whole saliva and oral fluid samples using commercial EIA was evaluated using different transport buffers (Fig. 1), type of whole saliva (Fig. 2), and volume of sample in assay (Fig. 3). The mean optical density (OD) value among paired negative serum samples was 0.017 ± 0.026 and among positive serum samples was 2.274 ± 1.453. No significant differences were observed among different types of buffer ($P = 0.719$), so PBS/BSA 0.5% was chosen as the OD values were closer to the OD values among serum samples. There was no significance among different fractions of whole saliva; therefore, whole saliva was employed ($P = 0.100$). When the volume of the sample was evaluated, no difference was also observed ($P = 0.07$ for whole saliva and $P = 0.10$ for oral fluid) and for that reason the same volume determined by the manufacturer was employed to facilitate sample analysis (150 µl).

The period of time and temperatures of whole saliva or oral fluid samples with conjugate incubation were also evaluated. OD values among whole saliva or oral fluid samples were more related to OD values in serum samples when the incubation period was extended to 18 ± 2 hr at room temperature instead of 90 min at 37°C (Fig. 4). Differences were significant for whole saliva, $P = 0.003$ and not significant for oral fluid $P = 0.06$.

As whole saliva or oral fluid samples are not routinely used to screen patients, there are no standard guidelines to calculate the cut-off absorbance value for these specimens. To calculate the CO value, three methods were employed. The ROC method (CO3) gave the best results. Cut-off values for oral fluid and whole saliva were 0.041 and 0.098, respectively. Using the AUROC,

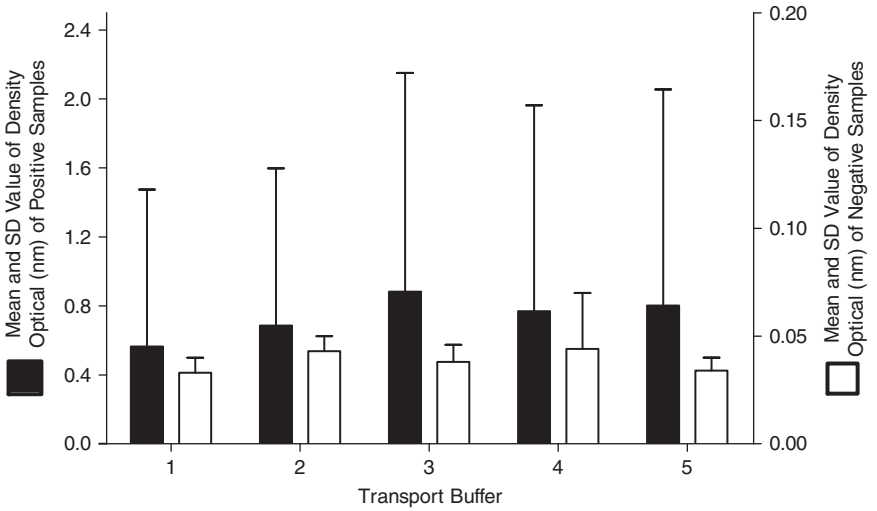

Fig. 1. Optical Density (OD) mean values (X ± SD) obtained in saliva samples according to transport buffer. Transport Buffers: (1) PBS pH 7.2; (2) PBS/Tween 20 0.05%; (3) PBS/Tween 20 (0.05%)/Sodium azide (0.005%); (4) PBS/Tween 20 (0.2%)/BSA 5%; (5) PBS/BSA 0.5%. OD mean value among paired negative serum samples was 0.017 ± 0.026 and among positive serum samples was 2.274 ± 1.453. Differences were not significant ($P = 0.719$).

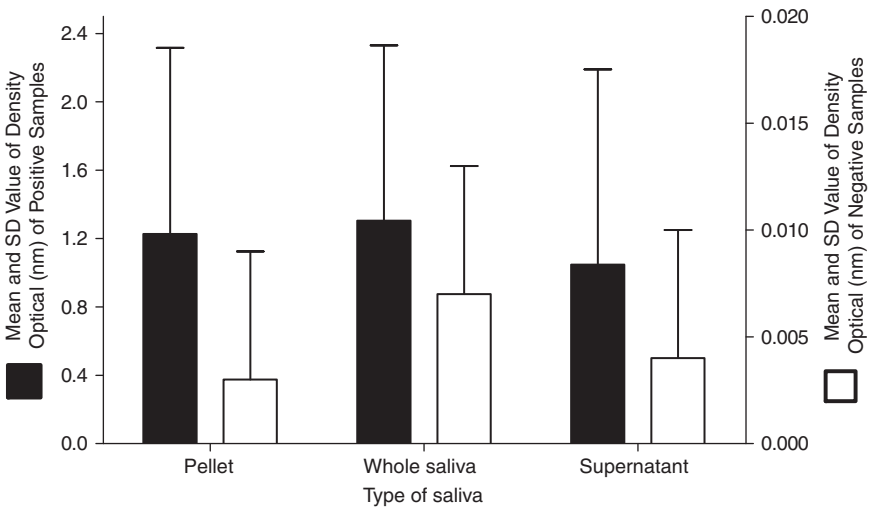

Fig. 2. Optical Density (OD) mean values (X ± SD) obtained according to type of saliva (pellet, whole saliva, supernatant). OD mean value among paired negative serum samples was 0.017 ± 0.026 and among positive serum samples was 2.274 ± 1.453. Differences were not significant ($P = 0.100$).

quality parameters, such as accuracy and PPV and NPV, were significantly improved, providing values superior to 89% for both specimens (Table 1).

Using ROC curve (CO3), HBsAg was detected in 40 oral fluid and 44 whole saliva samples out of 47 paired positive serum specimens and not detected in 64 oral fluid and 63 whole saliva samples out of 68 matched negative sera samples by ELISA assay (Table 2). Overall, the agreement between results obtained in serum and saliva specimens was excellent according to the Kappa index (κ: 0.80 for oral fluid and κ: 0.87 for whole saliva). Using the optimized protocol, sensitivities of whole saliva and oral fluid were 93.6 and 85.1%, respectively, whereas specificities of whole saliva and oral fluid were 92.6 and 94.1%, respectively.

Salivary assays showed excellent reproducibility as no discordant result was obtained among whole saliva and oral fluid by testing the samples in the optimized ELISA over a 3-day period and positive results were obtained on both samples containing until 15,000 UI/ml of HBsAg.

DISCUSSION

Saliva has been suggested as a convenient specimen for detection of antibodies for various infectious

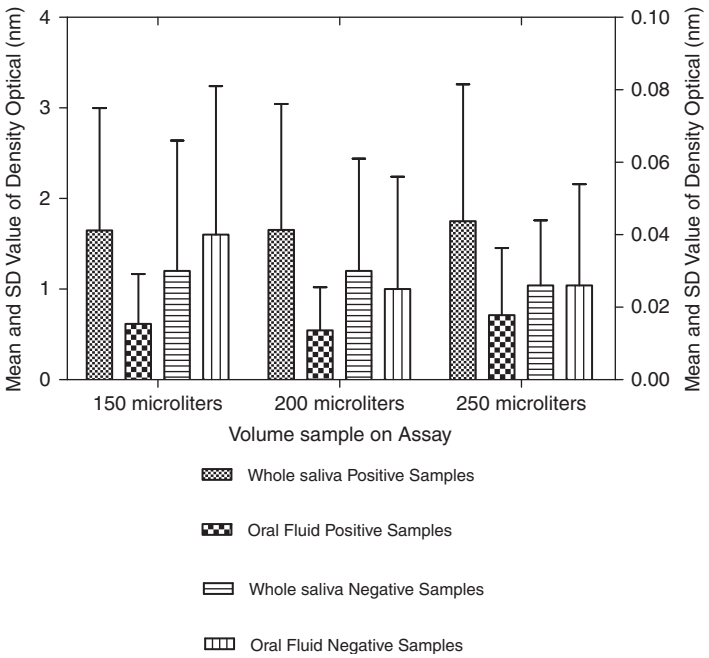

Fig. 3. Optical Density (OD) mean values (X ± SD) obtained according different sample volume on assay. OD mean value among paired negative serum samples was 0.017 ± 0.026 and among positive serum samples was 2.274 ± 1.453. Positive and negative samples results were plotted on left and right *Y*-axis, respectively. Differences were not significant (whole saliva, $P = 0.07$ and oral fluid $P = 0.10$).

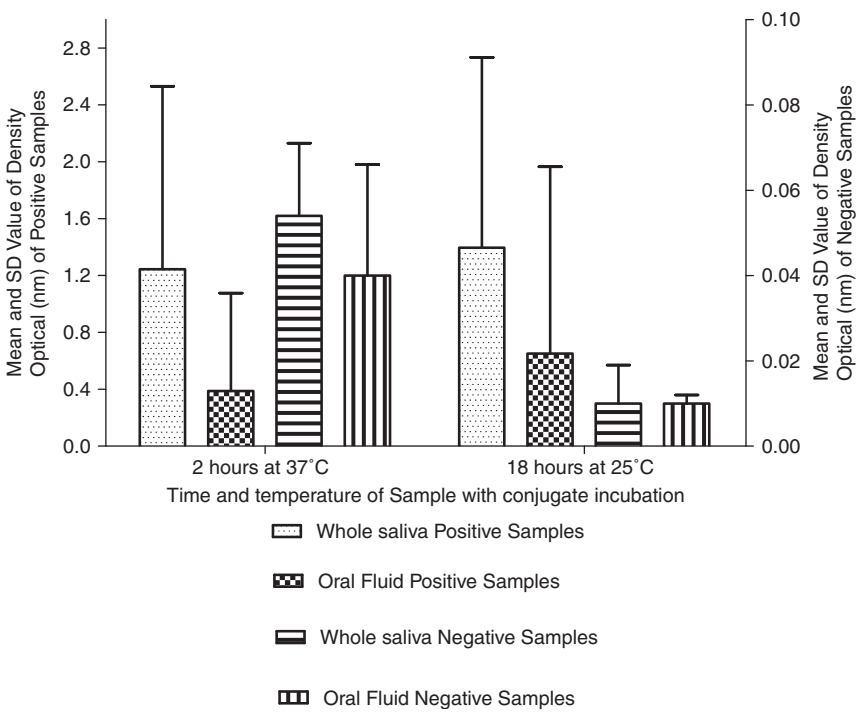

Fig. 4. Optical Density (OD) mean values (X ± SD) obtained among whole saliva and oral fluid according different period and temperature of sample with conjugate incubation. OD mean value among paired negative serum samples was 0.017 ± 0.026 and among positive serum samples was 2.274 ± 1.453. Positive and negative samples results were plotted on left and right *Y*-axis, respectively. Differences were significant for whole saliva, $P = 0.003$ and not significant for oral fluid $P = 0.06$.

TABLE 1. Accuracy Indices for Different Methods of Calculation Cut-off Absorbance for HBsAg Test on Oral Fluid and Whole Saliva

Cut-off value	Specimen	Absorbance value	% sensitivity (95% IC)	% specificity (95% IC)	PPV (95% IC)	NPV (95% IC)	Accuracy %
CO1	Oral fluid	1.0	85.1 (71.7–93.8)	92.6 (83.6–97.5)	88.8 (75.9–96.2)	90 (80.4–95.8)	89.5
	Whole saliva	1.0	93.6 (82.4–98.6)	80.8 (69.5–89.4)	77.1 (64.1–87.2)	94.8 (85.6–88.9)	86.0
CO2	Oral fluid	2.559	63.8 (48.5–77.3)	98.5 (92.0–99.9)	96.7 (92.0–99.9)	79.6 (69.5–87.7)	84.3
	Whole saliva	4.432	80.8 (66.7–90.8)	94.1 (85.6–98.3)	90.4 (77.3–97.3)	87.6 (77.8–94.2)	88.6
CO3	Oral fluid	0.041	85.1 (71.7–93.8)	94.1 (85.6–98.3)	90.9 (78.3–97.4)	90.1 (80.7–95.9)	90.4
	Whole saliva	0.098	93.6 (82.4–98.6)	92.6 (83.6–97.5)	89.8 (77.7–96.6)	95.4 (87.2–99.0)	93.0

PPV: Positive Predictive Value; NPV: Negative Predictive Value; Accuracy: TP+TN/TP+TN+FP+FN TP: True Positive; True Negative; False Positive; False Negative. CO1: Cut-off value determined by the manufacturer; CO2: Cut-off value determined by 3 standard deviations above the mean saliva absorbance of HBV seronegative samples (mean+3SD); CO3: Cut-off value determined by the area under receiver operating characteristic (AUROC) curve analysis for saliva absorbance values.

TABLE 2. Results of the Modified ELISA Test to Detect HBsAg in Oral Fluid and Whole Saliva in Paired Positive and Negative HBsAg Serum Specimens

Sample	Oral fluid			Whole saliva		
Serum	Positive	Negative	Total	Positive	Negative	Total
Positive	40	7	47	44	3	47
Negative	4	64	68	5	63	68

diseases. Hepatitis viruses occupy a prominent place in this list with numerous studies reporting a favorable sensitivity of saliva for hepatitis marker detection (9,10–12). Saliva sample collection is easy, cheap, and noninvasive and does not require specialized transportation measures. Therefore, saliva has the potential to replace serum/plasma-based screening in community-based seroprevalence studies. This study aimed to adapt a routinely used immunoassay for salivary HBsAg detection.

Different components of saliva can be used for the detection of viral markers, such as stimulated or unstimulated whole saliva, glandular duct saliva, and GCF. Oral fluid contains mainly GCF and is collected by using specialized collection devices such as the commercial device used in this study (Salivette, Sarstedt). These devices are optimized to collect mainly GCF and minimize the amount of the glandular saliva in the specimen. Whole saliva and Salivette devices were chosen due to the low cost of both methods. Salivette device is one of the cheapest devices sold on the Brazilian market.

In this study, oral fluid and whole saliva were evaluated to detect HBsAg, and it was possible to detect HBsAg with both specimens. Whole saliva and oral fluid samples were employed to detect HBsAg (3,11,16–19,21). The HBsAg detection was higher in whole saliva, probably because this sample is more representative of the oral milieu as it contains secretions of the major and minor salivary glands (13). Moreover, cotton swabs like Salivette absorb fluid very efficiently and a small liquid volume is quickly dispersed across a large surface area of the intertwined cotton fibers. When the surface area of the cotton used is large relative to the sample volume available to be absorbed, the liquid can be so diffusely distributed in the fibers that despite centrifugation or pressure it is difficult to recover a sufficient test volume. Furthermore, these fibers can interfere in the immunoassay and give unspecific results (26).

The interference caused by filtering saliva through cotton could be due to either the (i) concentration of saliva due to the capture and retention of water molecules by the cotton, (ii) release of material from the cotton those cross-reacts or nonspecifically interferes with immunoassays, or (iii) binding and retention of molecules of interest by the cotton fibers. These potential problems should be considered when cotton absorbent materials are used to collect saliva (27).

The choice of the collecting system/assay combination depends on the ease of use of the collecting device, turnaround time of the assay, reliability of the results, and economical considerations. Based on the results of this study, whole saliva was most adequate to detect HBsAg on saliva samples due to low cost, easiest collection, and high accuracy of the assay. Whole saliva has already been used for HBV markers detection, such as HBV DNA, anti-HBc, or HBsAg, presenting high sensitivity and specificity (3,16,19,28). However, most of

the participants in this study reported that Salivette collection was more hygienic than whole saliva collection, so it is necessary to improve the assay quality parameters of the Salivette assay, such as the mode of collection. Another alternative is the use of a different device to detect HBsAg among oral fluid samples. Thieme et al. (29) and Piacentini et al. (30) reported the use of the Orasure device which gave 100% of sensitivity and specificity for HBsAg detection. Mortimer et al. (15) reported that reactions from positive specimens for measles collected by swab or into a pot were mostly slightly stronger than the reactions from Salivette specimens. Vyse et al. (14) evaluated three devices (Oracol, Omni-SAL, and Orasure) for rubella detection and showed that Oracol was the most suitable.

In this study, HBsAg were detected more frequently in the serum samples than whole saliva or oral fluid samples probably due to the low amount of HBV antigen in the saliva. Moreover, saliva samples presenting negative results had positive paired serum samples showing high concentration of HBsAg (OD value higher than 3.0). This situation demonstrates that serum HBsAg concentration was not associated to saliva HBsAg detection. Further studies should be carried out to explain this observation.

To detect HBsAg in saliva samples, optimization of numerous parameters should be carried out. In this study, sample and conjugate incubation temperature and duration were modified to improve OD values as was done in another study (31). Another approach for assay optimization was the use of a modified cut-off value for whole saliva and oral fluid samples. Three methods were employed to calculate cut-off values such as demonstrated in previous studies (7,8,32–34). In our study, AUROC analysis method (using CO3) provided reasonable sensitivity and specificity desirable for a screening assay and has been suggested as an effective alternative to other methods to estimate the cut-off absorbance (25).

Using an optimized protocol, accuracy of the salivary HBsAg assay was more than 89% for whole saliva and oral fluid samples. Both specimens could be used to detect HBsAg and researchers should establish which specimens is the best choice according to the laboratory facilities available. Oral fluid presents low risk of contamination compared with whole saliva, but whole saliva is cheaper than oral fluid collection as there is no need to use any commercial device.

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