A NON-INVASIVE ASSESSMENT OF HEPATITIS B VIRUS CARRIER STATUS USING SALIVA SAMPLES

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Abstract. A non-invasive testing method to determine hepatitis B virus (HBV) carrier status in pregnant women was evaluated. Paired serum and saliva samples were collected and assessment of hepatitis B markers were performed. Of the 502 women enrolled, 5.6% (28/502) of their sera were found to be positive for HBV surface antigen (HBsAg). Assessment of 28 HBsAg seroreactive and 200 HBsAg sero-non-reactive paired saliva samples showed that 17 saliva contained HBsAg. Fourteen of the saliva reactive samples were matched to the serum reactive samples (50% sensitivity); and 3 saliva samples were positive for HBsAg among 200 subjects seronegative for HBsAg (98.5% specificity). Seven of the 28 HBsAg positive sera were found to be reactive for HBV envelope antigen (HBeAg) (25%). One of seven HBeAg seroreactive and 16 HBeAg seronegative paired saliva samples tested were non-reactive for HBeAg. This report found a non-invasive saliva testing method to be a possible alternative approach for determining chronic HBV carrier status if the sensitivity of the test can be improved.

INTRODUCTION

Worldwide, chronic HBV infection is a leading cause of chronic hepatitis, cirrhosis, and hepatocellular carcinoma (Bloom *et al*, 1993). As such, HBV is a major cause of morbidity and mortality, especially in developing countries where the prevalence of chronic infection is highest. Numerous incidence studies have been conducted to evaluate perinatal transmission of HBV from chronically infected, HBsAg-positive mothers to infants at birth. These studies have consistently indicated that chronic infection follows in a very high percentage (80-90%) of infants born to extremely infectious HBsAg/HBeAg-positive carrier mothers and follows in a much lower percentage of infants born to less infectious HBsAg-positive/HBeAg-negative mothers (Beasley *et al*, 1977, 1981; Hyams, 1995).

Chronic HBV infection can be prevented in most infants born to carrier mothers who are HBeAg-negative by giving the hepatitis B vaccine alone. However, in children born to highly infectious mothers with HBeAg, hepatitis B immune globulin (HBIG) is required, in addition to the hepatitis B vaccine, to prevent transmission. Unfortunately, both hepatitis B vaccine and HBIG are very expensive, and it is not possible in many developing countries to vaccinate all newborns against HBV infection regardless of maternal infection. However, by testing pregnant mothers prior to delivery for HBsAg and HBeAg, the infants most at risk of chronic infection can be identified and vaccination targeted at this group, particularly infants requiring additional HBIG prophylaxis.

A cheap and non-invasive method for testing pregnant women for HBV infection would be invaluable in developing countries where contaminated needles and syringes are sometimes re-used and patients are reluctant to provide blood samples. Use of a non-invasive testing method would prevent unnecessary infectious disease transmission to mothers and their children and would facilitate the prevention of HBV transmission to infants by identifying carrier mothers. In addition, non-invasive test methods could prove to be cheaper and safer in the diagnosis of acute and chronic hepatitis in different groups of patients.

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Two major oral fluid collection systems have been proposed. One includes the capture of saliva (Frerichs *et al*, 1992; de Azevedo Neto *et al*, 1995) and the other buccal cavity fluid (Soto-Ramirez *et al*, 1992; Thieme *et al*, 1992). Both methods require clinical samples that contain similar antibody and infectious agent antigen to that found in blood. This study evaluates the Saliva Diagnostic Systems (SDS) Inc (Vancouver, WA) saliva collection device to assess the chronic hepatitis status of pregnant women.

MATERIALS AND METHODS

Clinical specimens

The study population included 502 women in varying stages of pregnancy who attended the prenatal clinic at Dr Jose Fabella Memorial Hospital, Manila, Philippines. After providing informed written consent, a 10 ml volume of venous blood and a 1 ml saliva sample were obtained from each participant to determine the presence of HBV serologic markers. The samples were coded without personal identifiers and analyzed blindly at the US Naval Medical Research Unit No. 2 (NAMRU-2), Jakarta, Indonesia.

Collection of saliva samples

OMNI-SAL sterile saliva collection devices supplied by SDS Inc, were used as directed by the manufacturer. Briefly, a collection pad was placed into the mouth, beneath the tongue and behind the teeth, of each volunteer. With the mouth closed, the pad absorbed saliva for approximately 2 minutes until it was well saturated (wet and limp). Subsequently, the pad was removed and inserted into a tube containing 1 ml of transport buffer, mixed and transferred to the laboratory. Saliva eluate was separated from the pad and stored at -20°C until assayed.

Assays

All 502 serum samples were tested for HBsAg utilizing the commercial kit-Auszyme Monoclonal EIA procedure A (Abbott Laboratories, Abbott Park, IL) following the manufacturer's instructions. In addition, each sample positive for HBsAg was subsequently tested for HBeAg and anti-HBe [HBe (rDNA) EIA, Abbott Laboratories].

Saliva samples were assessed (blindly) by similar procedures to those described for serum samples above, unless otherwise stated. The Auszyme Monoclonal EIA procedure D was used for 228 saliva tested for HBsAg. This assessment included 28 HBsAg seroreactive and 200 randomly chosen HBsAg seronegative paired saliva samples. Auszyme procedure D was employed because it used an increased volume of conjugate (200 µl; instead of the 50 µl described in procedures A-C) which elevated the sensitivity for detecting HBsAg in saliva. For determination of the presence of HBeAg in saliva, one HBeAg seropositive and 16 HBeAg seronegative paired saliva had sufficient volume for testing. Also, eight seropositive and 10 seronegative anti-HBe paired saliva samples were assayed for anti-HBe. For the anti-HBe assays, the saliva were appraised utilizing 50-200 µl volumes, whereas the manufacture's pamphlet recommends the use of only 50 µl of serum for the same procedure. Increased volumes were used to try to increase the sensitivity of the anti-HBe test.

For the HBeAg, anti-HBe and HBsAg assays, seronegative saliva samples were employed in place of the kit negative controls to determine the cutoff values for reactivity. This was done because all saliva samples (200 µl) would have been considered reactive for the HBeAg and anti-HBe assays, regardless of seroreactivity, due to increased absorbance. The cutoffs determined by calculating the saliva seronegative control mean (NCx) for HBsAg were only slightly higher than those calculated with the kit negative controls, and therefore did not alter the outcome of these assays. For the anti-HBe assays, OD values that were less than or equal to (NCx-PCx)/2 were considered to be reactive. For HBsAg and HBeAg assays, values above NCx plus 50 or 60, respectively, were considered reactive. Only repeatedly reactive saliva or serum samples were considered positive.

RESULTS

HBsAg testing of 502 serum samples from pregnant Filipino women found 28 (5.6%) were reactive for HBsAg. Follow up assessment of the 28 HBsAg seropositive and 200 randomly selected HBsAg

seronegative paired saliva samples for HBsAg reactivity discovered that 17 saliva samples were positive for HBsAg. As shown in Table 1, fourteen saliva samples from 28 subjects seroreactive to HBsAg also were reactive to HBsAg (50% sensitivity), and three of the 200 seronegative paired saliva samples were falsely positive for HBsAg (98.5% specificity).

HBeAg presence in HBsAg reactive and non-reactive serum and saliva samples were subsequently assessed. Seven of the 28 HBsAg reactive sera were found to be reactive to HBeAg (25%). Of the seven reactive serum samples only one of the corresponding saliva samples was available for testing and this specimen was HBeAg non-reactive. None of 16 HBeAg seronegative saliva samples assayed, were found to be reactive for HBeAg.

Anti-HBe reactivity was assessed in 52 serum samples and 18 paired saliva samples. Twenty-six of the 52 serum samples tested were found to be anti-HBe reactive. All saliva samples assessed at the manufacture's recommended volume of 50 µl were negative for anti-HBe. However, when the sample volume was increased to 200 µl, all the samples appeared to be reactive, regardless of serum anti-HBe reactivity. These apparent false positive reactions were subsequently found to be negative when we modified the manufacturer's protocol to include negative control saliva samples as opposed to the negative controls included in the kit. Thus, the lack of sensitivity of the anti-HBe assay could not be overcome by increasing the volume of saliva tested.

DISCUSSION

In this study, 5.6% of blood samples obtained from pregnant women attending a prenatal clinic in Manila, Philippines, were found positive for HBsAg. In a large portion (50%) of the HBsAg seropositive women we also found HBsAg reactivity in the paired saliva samples (Table 1). Because of the potential transmission of HBV from HBsAg-positive mothers to their child at birth, the HBsAg marker of chronic hepatitis B infection provides a consequential signal to the health care provider for the appropriate use of intervention strategies (*eg* treatment with hepatitis B vaccine and HBIG).

The need for identifying HBV carriage and potentially preventing HBV infection in children is critical because this age group is most susceptible to chronic infection (Hyams, 1995). However, the ability to identify newborns at risk of HBV infection can be limited by the expense and potential danger of blood collection procedures used in developing countries. Therefore, it is important to find alternative methods for the identification of HBV carriage, and we believe that the saliva collection protocol described here may be useful, if the sensitivity of this method can be improved.

The SDS Omni-Sal saliva collection device was found easy to use, and the volunteers readily accepted the collection method. The inability to detect viral markers in all saliva of seroactive individuals analyzed using the Abbott commercial EIA kits, might be overcome in the future by modification of these kits. However, increasing the volume of saliva assessed did not enhance the sensitivity of the assays. We found that enlarging the saliva volume tested in the anti-HBe procedure from 50 to 200 µl only increased the observance of non-specific reactivity. It is unclear what the interfering substance was that resulted in the high OD values observed for all case and control saliva samples assayed.

Table 1

Sensitivity and specificity of saliva testing method for hepatitis B carrier status.

	+ HBsAg	-	+ HBeAg	-	+ Anti-HBe	-
Sera	28/502*	474/502	7/28	21/28	26/52	26/52
Saliva	14/28	3/200	0/1	0/16	0/8	0/10
Sensitivity	50%	n/a	0%	n/a	0%	n/a
Specificity	n/a	98.5%	n/a	100%	n/a	100%

* Number reactive samples/total number of samples tested.

Saliva testing for evidence of exposure to HBV (Heatcote *et al*, 1974; Feinman *et al*, 1975; Parry *et al*, 1987; Siddiqi *et al*, 1988; Parry *et al*, 1989; Thieme *et al*, 1992), HAV (Parry *et al*, 1987, 1989; Laufer *et al*, 1995; Thieme *et al*, 1992), HCV (Thieme *et al*, 1992), HIV (Archibald *et al*, 1986; Parry *et al*, 1987; Shoeman *et al*, 1989; Frerichs *et al*, 1992; Soto-Ramirez *et al*, 1992) and other pathogenic viruses (Parry *et al*, 1987; Perry *et al*, 1993; de Azevedo Neto, 1995) has been extensively evaluated. In these studies the obvious benefits for utilizing non-invasive techniques were found to include: 1) ease of use-because saliva collection devices do not require professionally trained personnel to perform the procedure; 2) increased compliance due to less pain and discomfort; 3) minimization of nosocomial infections due to contaminated needles and syringes; 4) increased number of samples collected and processed; and 5) decreased cost of specimen collection. These beneficial characteristics have ultimately led to the FDA approving the collection of oral fluid specimens (OraSure HIV-1 Oral Specimen Collection Device, Epitope Corporation of Beaverton OR) and a specific test used to analyze the specimens for the presence of antibodies to HIV (Oral Fluid Vironostika HIV-1 Microelisa System, Organon Teknika Corp, Durham, NC).

In conclusion, we have found that the inexpensive, non-invasive testing method described in this paper for laboratory diagnosis of chronic HBV infection (presence of HBsAg in saliva) to be a possible alternative procedure to that of serum testing, especially in populations in which individuals are reluctant to provide blood samples and/or there is a potentiality of re-use of blood collection devices.

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