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New, Ultrasensitive Enzyme Immunoassay for Detecting Vaccine- and Disease-Induced Hepatitis A Virus-Specific Immunoglobulin G in Saliva

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Although detection of disease-induced hepatitis A virus (HAV)-specific antibodies in saliva has been successfully utilized in a few epidemiological studies, available assays fail to detect lower salivary anti-HAV levels associated with vaccine-induced immunity. We present a new capture enzyme immunoassay which employs a three-layer antibody recognition system. Evaluation of paired saliva-serum specimens from 1,025 international travellers, 134 other volunteers, and 91 hepatitis A vaccine recipients demonstrated 99.6% (95% confidence interval, 98.4 to 99.9) specificity and 98.7% (95% confidence interval, 97.7 to 99.4) sensitivity of this salivary assay in differentiating between immune and susceptible individuals, compared with serum-based methods. We conclude that this assay is sufficiently sensitive for reliable detection of both vaccine- and infection-induced HAV-specific immunoglobulin G in saliva, even when corresponding anti-HAV levels in serum are very low (<1 IU/ml).

Recently, there has been considerable interest in saliva as a more easily obtained specimen than blood for evaluating humoral immunity to a number of infectious agents (1, 9, 11, 13, 14, 16, 17). Testing for specific immunoglobulin G (IgG) antibodies in saliva could be especially useful in serosurveys, particularly in children or others where blood specimen collection may be difficult. Hepatitis A virus (HAV) is one of the first pathogens for which saliva-based antibody assays were established (9, 12). These were capture radioimmunoassays (RIAs) which detected virus-specific IgM and IgG. Detection of HAV-specific antibodies in saliva by these methods was reported to be accurate in hepatitis A diagnosis and in differentiating between HAV-immune and -susceptible individuals, provided that immunity followed natural infection, in which HAV antibody levels are high (10, 12). Conventional enzyme immunoassays (EIAs) designed for detection of HAV-specific antibodies in serum have been used to detect specific antibodies in saliva for confirmation of HAV infection (16). In this case, sensitivity was enhanced by using oral collection devices that stimulate transudation of immunoglobulins from the vascular space to saliva (16). However, both capture RIA and EIAs were insufficiently sensitive to detect immunity induced by inactivated HAV vaccine where serum anti-HAV levels tend to be at least 10 times lower than those resulting from HAV infection (6, 7, 18). Thus, the insensitivity imposed significant constraints on the monitoring of HAV vaccine-induced antibodies in saliva, as well as on the detection of threshold levels in serum from low responders. Here, we describe a new, ultrasensitive EIA which is amenable to the evaluation of vaccine- and disease-induced HAV-specific IgG antibodies in saliva. Also reported are data resulting from a field trial of this assay for the screening of HAV-specific IgG levels in recipients or potential recipients of inactivated HAV vaccine. The results indicate the high sensitivity and specificity of this method for salivary detection of specific antibodies in comparison with serologic methods and indicate great promise for more effective management of HAV vaccine delivery.

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MATERIALS AND METHODS

Study subjects. Study subjects included 91 adult participants in a trial of HAV vaccine from whom paired serum and saliva samples were obtained 4 weeks after the third dose of inactivated HAV vaccine (Havrix; SmithKline Beecham Biologicals). Study participants also included 1,025 international travellers seeking immunization advice at Vancouver Health Department pretravel clinics and 134 other volunteers (69 adult hospital and laboratory staff members and 65 teenage participants in a hepatitis B vaccine trial). A broad range of ages and ethnic backgrounds was represented. All subjects had a negative history of immunoglobulin administration or blood transfusion within 6 months before entering the study. With the exception of participants in the HAV vaccine trial, all remaining subjects denied earlier hepatitis A vaccination.

Specimen collection. Paired saliva and serum samples were collected from all study participants. Vaccinees and 22 volunteers produced whole saliva samples by salivating directly into large, open tubes. Saliva collection from travellers and the remaining 112 volunteers was facilitated by the use of Salivette (Sarstedt Inc.) with a neutral insert (pad). Pads were held in the mouth until thoroughly saturated. Saliva was later recovered by centrifugation. All saliva and serum specimens were processed for storage on the day of collection, aliquotted in small cryovials, and stored at −70°C until tested.

Determination of HAV-specific antibodies in serum samples. All sera collected from travellers were screened for the presence of total anti-HAV by using the HAVAB IMX assay (Abbott Laboratories, Abbott Park, Ill.) at the Virology Laboratory of the B.C. Center for Disease Control, Vancouver, British Columbia, Canada. Sera from volunteers and vaccinees were tested at the Vaccine Evaluation Center Laboratory for total anti-HAV content by using the Heprofile Anti-HAV assay (ADI Diagnostics, Rexdale, Ontario, Canada) in accordance with the manufacturer's directions. In this competitive-type EIA, anti-HAV from test samples competes with peroxidase-labelled anti-HAV for an HAV antigen coating in microplate wells. Classification of samples as negative or positive for anti-HAV depends on the absorbance observed in wells with test samples in comparison with cutoff values based on positive and negative controls included in each run. Positive results obtained with the Heprofile assay were confirmed with the HAVAB IMX assay or by measurement of total anti-HAV content by a quantitative EIA (2) developed by SmithKline Beecham Biologicals. This inhibition-type enzyme-linked immunoassay is calibrated against a World Health Organization standard, and anti-HAV titers are expressed in milli-international units per milliliter. The latter was used for all specimens from vaccinees.

Determination of HAV-specific IgG antibodies in saliva specimens. All saliva samples were tested for HAV-specific IgG antibodies with a new capture EIA

performed as follows. To capture human anti-HAV, the wells of 96-well, flat-bottom polystyrene microplates (Immulon 2; Dynatech Inc., Chantilly, Va.) were first coated overnight with $F(ab)_2$ fragments of donkey anti-human IgG (Jackson Immuno Research Laboratories, West Grove, Pa.) at a concentration of 1.2 μg/ml in carbonate-bicarbonate buffer (pH 9.6). The wells were subsequently blocked for 1 h with 1% (wt/vol) bovine serum albumin (BSA; Sigma Chemical Company, St. Louis, Mo.) in phosphate-buffered saline (pH 7.4) containing 0.05% (vol/vol) Tween 20 (PBS-T). For each specimen tested, four wells were allocated. Saliva specimens diluted 1:4 in PBS-T containing 0.5% BSA were incubated on the plate for 1.5 h to allow IgG class antibody capture. Of the wells assigned to each specimen, two subsequently received HAV antigen (kindly supplied by SmithKline Beecham Biologicals, Rixensart, Belgium) diluted to 432 enzyme-linked immunosorbent assay units/ml in PBS-T containing 0.5% BSA while the remaining two received only buffer to serve as antigen negative control wells for nonspecific binding. HAV antigen was incubated overnight to allow adherence to wells containing HAV-specific IgG. To detect captured HAV-specific IgG complexes, all microplate wells were incubated for 1.5 h with a 0.025-μg/ml dilution (in PBS-T containing 0.5% BSA) of an HAV-specific monoclonal antibody (Clone K3.4C8; Commonwealth Laboratories, Parkville, Australia), followed by exposure of all wells for another 1.5 h to alkaline phosphatase-conjugated $F(ab)_2$ fragments of donkey anti-mouse IgG (heavy and light chains; Jackson Immuno Research Laboratories, West Grove, Pa.) diluted to 0.2 μg/ml in PBS-T containing 0.5% BSA. The amount of bound alkaline phosphatase conjugate was then determined spectrophotometrically with an enzyme-linked immunosorbent assay amplification system (Life Technologies Inc., Gaithersburg, Md.) based on the reduction of iodonitrotetrazolium by NADH (8). This signal-enhancing method was used in accordance with the manufacturer's instructions and required 15 min of incubation with the substrate. When the A_{490} in the positive control wells reached 1.4, the amplification phase was stopped by addition of 50 μl of 0.3 M sulfuric acid per well.

All incubations (including antibody coating) were performed at room temperature. Between reagent additions, microplate wells were washed extensively with PBS-T or 0.05 M Tris/HCl buffer (pH 7.5) to remove unbound constituents. Washing was facilitated by an LP 35 automated microplate washer (Diagnostic Pasteur, Montreal, Quebec, Canada).

For each specimen tested, the ratio of the absorbance observed in wells exposed to HAV to the absorbance in antigen control wells was calculated. A sample was considered to be positive for HAV-specific IgG when the ratio exceeded an established cutoff value. This value was calculated as the mean plus 2 standard deviations of ratios determined with salivary specimens obtained from 200 study participants determined to be seronegative.

Criteria for determination of the sensitivity and specificity of the capture EIA for detection of HAV-specific antibodies in saliva. The clinical usefulness of a saliva-based assay for HAV-specific IgG is a function of its ability to differentiate between immune and susceptible individuals. Since serologic tests are recognized to be 100% sensitive and specific in this capacity, we used them as the "gold standard" for comparison with our salivary antibody test. We estimated that to detect 95% sensitivity and specificity with a precision of ±2% would require the evaluation of paired saliva and serum specimens from at least 360 seropositive and 360 seronegative individuals. Sensitivity of the salivary EIA was calculated as the percentage of positive saliva test results among individuals found to be seropositive. Conversely, specificity was expressed as the percentage of negative saliva test results among subjects determined to be HAV seronegative. Absolute confidence intervals were calculated.

RESULTS

Anti-HAV antibody in serum and saliva of HAV vaccinees. All 91 HAV vaccine recipients were found to be seropositive for anti-HAV by both Heprofile and quantitative EIA. The lowest level of total virus-specific antibody in serum observed in this group was 80 mIU/ml, while the highest value was 35,100 mIU/ml. Thirty vaccinees (33%) had antibody levels in serum greater than 6,000 mIU/ml, while among the remaining 61 vaccinees, 15 individuals had antibody levels in serum lower than 2,000 mIU/ml. Ninety-one (100%) of the corresponding saliva samples from seropositive vaccinees were also found to be positive for HAV-specific IgG antibody by capture EIA.

Anti-HAV antibody in international travellers. Paired serum and saliva specimens from 1,025 travellers were tested for HAV-specific antibodies. Of the 327 travellers found by HAVAB to be seropositive for anti-HAV of all immunoglobulin classes, 325 (99.4%) also tested positive for HAV-specific IgG in saliva. Among the 698 seronegative subjects, salivary testing for HAV-specific IgG was also negative in 689 cases (98.7%). In the nine discrepant saliva samples, the observed signal-to-background ratios were marginally above the cutoff point.

TABLE 1. HAV-specific antibodies in paired saliva-serum specimens[a]

Result of HAV antibody test of:		No. of:			Total
Serum	Saliva	Vaccinees[b]	Travellers[c]	Volunteers[d]	
+	+	91	325	33	449
+	−	0	2	0	2
−	−	0	689	100	789
−	+	0	9	1	10
Total		91	1,025	134	1,250

[a] Total HAV-specific antibodies were determined in serum, and HAV-specific IgG was determined in saliva.
[b] Samples were collected 4 weeks after administration of the third dose of HAV-inactivated vaccine.
[c] Attendees of Vancouver public health travel clinics.
[d] Volunteer participants were hospital and laboratory staff members or teenage participants in a hepatitis B vaccine trial.

Anti-HAV antibody in volunteers. Among the 134 volunteers, 33 were seropositive and 101 were seronegative for total anti-HAV antibody by Heprofile. Only one disconcordant salivary test result was observed. This occurred in a seronegative individual whose saliva tested weakly positive for HAV-specific IgG.

In summary, the results in Table 1 show that 449 of 451 seropositive study subjects also had a positive salivary test, indicating a 99.6% (95% confidence interval [CI], 98.4 to 99.9) sensitivity for the capture EIA in determining HAV-specific IgG in comparison with conventional serologic assays for total specific HAV immunoglobulins. Among the 799 seronegative subjects, 789 also had a negative saliva test, indicating a specificity of 98.7% (95% CI, 97.7 to 99.4) for the salivary antibody EIA. The sensitivity and specificity of the capture EIA as determined in comparisons of specimens from unimmunized travellers were 99.4% (95% CI, 97.8 to 99.9) and 98.7% (95% CI, 97.6 to 99.4), respectively. Among tests performed on serum and saliva specimens from both travellers and volunteers, sensitivities and specificities were calculated to be 99.4% (95% CI, 98 to 99.9) and 98.7% (95% CI, 97.7 to 99.4), respectively.

DISCUSSION

Salivary antibodies of the IgG class originate mainly in plasma and transude from capillaries in the tissue lining the gingival crevice. Actual concentrations of IgG in oral fluid fluctuate with the changing mixture of saliva and crevicular fluid (13) and are believed to be approximately 800- to 1,000-fold lower than those found in serum (3, 13, 15). Thus, reliable detection of specific IgG in saliva requires an extremely sensitive assay. Most immunoassays designed for serum antibody determination lack sufficient sensitivity for this purpose. However, changes in incubation time, specimen dilution factors, or ranges of reference curves occasionally improve the lower limits of detection of such methods. Also, oral fluid collection devices which use a hypertonic solution to increase transudation of immunoglobulins can increase the concentration of specific IgG in oral fluid specimens (4).

The clinical usefulness of a saliva-based test for HAV-specific IgG depends more on its ability to differentiate between HAV-immune and -susceptible individuals than on its ability to measure exact concentrations of a specific antibody in saliva. The assay format described here was designed to differentiate

between immune and nonimmune samples rather than to be quantitative. When calibrated against the World Health Organization's international reference serum, our assay had a lowest detection limit of around 0.0015 mIU/ml. Data on the lowest limits of detection of other published salivary assays are not available.

The EIA described here is a capture assay in which a class-specific antibody, used to coat the solid phase, immobilizes a representative sample of a given class of antibody from a specimen. Antigen is then added to interact with any specific antibody that has been captured. The amount of bound antigen then reflects the proportion of specific antibody present in the captured representation. This principle was successfully applied to the measurement of HAV-specific antibodies in serum (5) and for RIAs designed to detect specific IgG or IgM antibodies in saliva (9). These RIAs, although sufficiently sensitive to detect salivary HAV-specific IgG in cases of disease-induced immunity, were not useful in detecting the substantially lower levels of specific IgG found in vaccinees. The inadequacy of these assays for salivary antibody detection was particularly evident when corresponding levels of anti-HAV in serum were lower than 9,000 mIU/ml, as is in most vaccinees (6, 7).

Achieving the sensitivity required to detect the low levels of HAV-specific IgG in saliva from vaccinees or previously infected individuals with low levels of specific IgG in serum required two additional steps which make this EIA distinct from other available immunoassays. First, instead of using enzymatically or radioisotopically labelled HAV antigen or HAV-specific antibody, this assay employs a three-layer antibody detection system. This includes the use of unlabelled HAV antigen, followed by a highly specific murine anti-HAV monoclonal antibody which is subsequently detected by enzyme-conjugated polyclonal anti-mouse IgG. Second, the sensitivity is further enhanced by the use of an enzyme-linked immunosorbent assay amplification system based on tetrazolium reduction by NADH at the stage of enzymatic reaction (8). Consequently, detection of anti-HAV in saliva was possible even in cases with corresponding antibody levels in serum lower than 0.1 IU/ml.

Data presented here indicate that saliva-based testing for IgG class HAV-specific antibody with the capture EIA is almost as accurate in identifying susceptible and immune individuals as conventional serum-based testing for total anti-HAV immunoglobulin levels. It demonstrated 99% sensitivity and specificity in tests of paired specimens from 1,250 individuals with a broad range of specific antibody levels in serum. Test characteristics were determined by using paired (saliva and serum) specimens from three distinct groups, although international travellers constituted the majority (82%) of the study participants. This group appears to be ideal for test validation, as in developed parts of the world, travellers to areas where HAV is endemic or epidemic are likely to be the largest group potentially requiring screening for preexisting immunity to HAV. The sensitivity and specificity of the salivary assay observed exclusively in travellers were not different from observations in a larger group consisting of travellers and other volunteers, who were mainly medical personnel. Inclusion of HAV vaccine recipients provided a particular challenge to test sensitivity. The group we studied was assessed shortly after completion of the vaccine series and specific antibody levels in serum are likely to wane with time after vaccination, possibly affecting antibody detection in saliva.

A flaw in the study design was lack of uniformity in the test methods applied to sera. However, the sensitivities of all of the serum-based methods used in this study were comparable and very close to 100%. As an additional safeguard, however, a substantial number of serum specimens found to be positive by one method were retested by another method and no discrepancies were encountered. The methods of saliva collection from vaccinees differed, but this did not appear to influence test sensitivity.

Since our salivary EIA detects antibodies of the IgG class only and the serum-based assays detected total anti-HAV of all immunoglobulin classes (IgG, IgM, and IgA), the few false-negative salivary test results observed might reflect the presence in serum of HAV-specific IgM and/or IgA without IgG. This, however, is unlikely since our capture assay, when applied to the "positive" sera in question, detected specific IgG in all of them (data not shown). False-positive salivary test results might be due to nonspecific interaction between the HAV antigen and saliva elements nonspecifically bound to the components used to capture class-specific antibodies. Alternatively, these discrepancies might simply represent inaccuracy (false negatives) of the serum-based assay used as the reference. The latter is more likely, since specific IgG was also present in all of the sera which correspond to false-positive saliva specimens when our capture assay was applied to them (data not shown). Parry et al. (10) also reported low rates of false positivity and negativity after natural HAV infection, but their salivary RIA also identified a substantial proportion of equivocal cases. The capture EIA presented here made a clear-cut distinction in all cases.

In conclusion, direct testing of saliva samples for HAV-specific IgG with a new capture EIA provides a useful alternative to serum-based assays for discriminating between HAV-immune and nonimmune individuals. The convenience, accuracy, and completely noninvasive nature of this method make it an attractive tool to assist the rational utilization of the newly available hepatitis A vaccines. It can help in the selection of nonimmune candidates for vaccination and in the monitoring of vaccine-induced immunity. Despite our encouraging preliminary results, the latter application requires further systematic investigation. Additionally, this assay may be effectively used in epidemiological surveys of the HAV immune status of selected at-risk populations, such as travellers, children attending day care centers, and residents in communities with poor sanitation facilities.

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