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An A-ELISA to Detect Hepatitis A Virus in Estuarine Samples

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An amplified enzyme-linked immunosorbent assay (A-ELISA) for detecting and quantifying hepatitis A virus in estuarine water samples is described. The test was five times more sensitive than a standard ELISA and at least two times more sensitive than radioimmunoassay. Test sensitivity was unaffected by the procedures used to concentrate the virus in estuarine samples or by the presence of humic and tannic acids in test samples. Nonspecific reactions were not encountered with a number of enteroviruses or with a rotavirus. A high sensitivity and specificity combined with speed, low cost, and freedom from radiolabels made the A-ELISA useful for detecting hepatitis A virus in environmental samples. The virus was detected in 3 of 20 estuarine water samples examined by A-ELISA.

Hepatitis A virus (HAV) is an important water- and food-transmitted pathogen (19). Outbreaks of HAV-associated hepatitis have occurred after ingestion of fecally polluted drinking or recreational water and consumption of raw or inadequately cooked, polluted shellfish (3, 9, 17). The first demonstration of HAV in feces was accomplished by immunoelectron microscopy (6). Subsequently, a number of detection methods of greater simplicity and sensitivity have been developed.

The solid-phase radioimmunoassay (SPRIA) is a sensitive test (11, 13). The enzyme-linked immunosorbent assay (ELISA) is as sensitive and more convenient, since it eliminates the need for radiolabels (18). The sensitivities of both the ELISA and SPRIA procedures have been increased through the use of monoclonal antibodies (MAbs) (2). In addition, fluorogenic substrates (14), ultrasensitive enzymatic radioimmune methods (12), and biotin-avidin indicator systems have been used to increase the sensitivity of detecting a positive result in tests with a number of viruses (1, 7, 10). Increased sensitivity of an ELISA can be realized from incorporation of a biotin-avidin system with the potential for amplification of antibody receptors due to multiple site binding. An amplified ELISA (A-ELISA) developed for detection of herpes simplex virus has a sensitivity comparable to that obtained by plaque assay of an infectious virus (1). An A-ELISA developed for detection of Norwalk virus has a sensitivity similar to or greater than that of the SPRIA (7). In this paper we report the development and evaluation of an A-ELISA to detect HAV in estuarine samples. We show that the test has a detection sensitivity two to five times greater than that of a SPRIA or a standard ELISA.

The HM-175 strain of HAV was used throughout these studies. This strain, adapted to growth in African green monkey kidney (AGMK) cells, was kindly provided by Robert Purcell, National Institutes of Health, Bethesda, Md. The development of persistently infected cultures and the recovery, concentration, and purification of the virus have been described elsewhere (14, 21). Roller bottle cultures of persistently infected AGMK cells were harvested at 24 days postseeding by several cycles of freezing and thawing. The virus was purified by chloroform extraction and cesium-chloride density gradient centrifugation (14).

Four mouse anti-HAV MAb preparations, in the form of mouse ascites fluids, were generously supplied by Emilio Emini, Merck Sharp & Dohme Research Laboratories, West Point, Pa. The ascites fluids were clarified by centrifugation at 14,000 × *g* for 10 min. Immunoglobulins were precipitated by 50% saturated ammonium sulfate, and anti-HAV-specific immunoglobulin G (IgG) was purified by column chromatography on protein A-Sepharose (4). Of the four antibodies, the MAb used was selected as the one that possessed the greatest degree of reactive affinity with purified HAV in radioimmunoassay (RIA) and blocking antibody tests. Polyclonal human anti-HAV serum (PAb), kindly provided by F. Blaine Hollinger, Baylor College of Medicine, Houston, Tex., was concentrated by saturated ammonium sulfate precipitation, and the IgG fraction was obtained by ion-exchange chromatography on DEAE-cellulose (5). Nonfat dry milk (Carnation Co., Los Angeles, Calif.) at a concentration of 2% (wt/vol) was used as a blocking reagent, carrier, and wash solution (15).

The monoclonal anti-HAV (IgG MAb) was labeled with biotin by the procedure of Guesdon et al. (10). Biotinyl-*N*-hydroxysuccinimide ester (0.1 M in dimethyl formamide) was combined with the IgG MAb (1 mg/ml in 0.1 M $NaHCO_3$, pH 8) at a molar ratio of 5:1 (biotinyl-*N*-hydroxysuccinimide ester/free amino groups of IgG). The mixture was incubated for 4 h at room temperature with continuous stirring and then dialyzed for 48 h at 4°C against several changes of 0.01 M phosphate-buffered saline (PBS) (pH 7.2). The final preparation was stored at −20°C until used. Alkaline phosphatase (Sigma Chemical Co., St. Louis, Mo.) was coupled to the IgG MAb by the method of Voller et al. (22). Briefly, 1.6 mg of IgG MAb per ml was mixed with 5 mg of alkaline phosphatase per ml at room temperature, and the solution was dialyzed for 18 h at 4°C against two changes of 0.01 M PBS (pH 7.2). Glutaraldehyde was added to a final concentration of 0.2% (vol/vol), and the solution was incubated for 3 h at room temperature with continuous mixing. The alkaline phosphatase-conjugated IgG was dialyzed for 18 h at 4°C against two changes of 0.01 M PBS (pH 7.2) and two changes of 0.05 M Tris hydrochloride (pH 7.2) containing 1 mM magnesium chloride. After dialysis, bovine serum albumin and sodium azide were added to concentrations of 1.0% and 0.02% (wt/vol), respectively. Human IgG PAb and mouse IgG MAb were labeled with ^{125}I by the iodogen (1,3,4,6-tetrachloro-3α,6α-diphenyl glycoluril) method (20). Briefly, 1 mg of iodogen was dissolved in 25 ml

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of chloroform, and 50 μl of this iodogen solution was dispersed in the bottom of borosilicate tubes. The chloroform was evaporated to dryness at room temperature to form a film of iodogen. Dry vials were stored at −20°C. PBS (0.01 M, pH 7.2, 35 μl) was added to the iodination vial, followed by $Na^{125}I$ (0.5 mCi, 5 μl) and IgG (10 μl, 1 mg/ml). The iodination was allowed to proceed for 2 min and was terminated by transferring the iodination solution to a vial containing 0.5 ml of PBS. The reaction mixture was run on a PD-10 column (Pharmacia, Inc., Piscataway, N.J.) to separate free ^{125}I from labeled IgG.

A-ELISA were performed in flat-bottomed polyvinyl microtiter plates (Dynatech Laboratories, Inc., Alexandria, Va.). Each well was coated for 16 h at 25°C with 75 μl of IgG MAb diluted in 0.01 M PBS (pH 8.5) containing 0.5 M NaCl. The wells were then treated with 200 μl of 2% lowfat milk per well for 1 h. After four rinses to remove the milk, 75 μl of an HAV sample (diluted in 0.01 M PBS, pH 7.2) or a concentrated field sample (in 0.15 M Na_2HPO_4, pH 7.2) was added, and the plates were incubated for 1 h at 37°C. The wells were rinsed four times, and 75 μl of biotinylated IgG MAb in 2% lowfat milk was added. After a 2-h incubation at 37°C, the wells were rinsed four times, 75 μl of a 1:200 dilution of alkaline phosphatase-conjugated streptavidin (Bethesda Research Laboratories, Inc., Gaithersburg, Md.) was added per well, and the plates were incubated for an additional 40 min at 20°C. Wells were rinsed five times, 100 μl of *p*-nitrophenylphosphate (1 mg/ml; Bio-Rad Laboratories, Richmond, Calif.) was added per well, and the plates were incubated at room temperature for 90 min in the dark. The results were analyzed at 414 nm with a Titertek ELISA reader, by using the substrate as blank. A sample was considered positive when the positive-to-negative ratio (P/N = OD sample/OD control, where OD is the optical density) was ≥2.1. The routine ELISAs were carried out as described for A-ELISA, except for the use of alkaline phosphatase-conjugated IgG MAb as a detector system. SPRIA were performed as described previously (13). Physical particle counts were made with a calibrated RCA model EMU 3F electron microscope as previously described (21).

Water samples were collected in the area of Seabrook, Galveston Bay, which receives secondarily treated sewage effluents. A water sample (378 liters) was collected in a 500-liter tank (Nalgene Labware Div., Nalge/Sybron Corp., Rochester, N.Y.), and the pH of the sample was adjusted to 3.5 with 1.0 N HCl. Aluminum chloride was added to a final molarity of 0.5 mM, and the sample was filtered sequentially through pleated filters (pore size, 3.0 and 0.45 μm; Filterite Corp., Timonium, Md.) at a flow rate of 10 liters per min. Adsorbed virus was eluted from the filters after 30 min of contact with 1 liter of 0.05 M glycine, pH 9.5, containing 10% tryptose phosphate broth and 3% beef extract. The eluate was collected by backflushing the filter under positive pressure, and the pH was adjusted to 7.2. The samples were transported on ice to the laboratory and stored at 4°C. Before analysis, the eluate was further concentrated by organic flocculation to a total volume of 10 to 20 ml (16).

Experiments were performed to optimize the A-ELISA for detection of HAV. A 4-μg/ml concentration of IgG MAb was found most effective for capturing HAV. The efficiency of MAb attachment to bound HAV was determined by competitive RIA. Human IgG PAb labeled with ^{125}I was added to wells containing HAV bound to IgG MAb. The MAb blocked up to 100% of the specific binding of the ^{125}I-labeled human IgG PAb. The optimal concentration of biotinylated MAb was determined to be 30 μg/ml. A greater

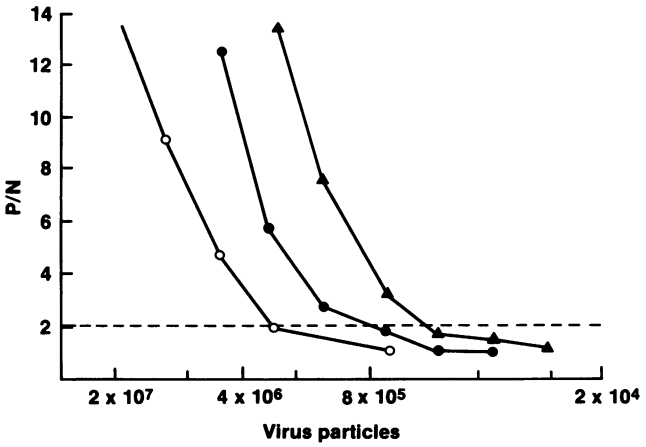

FIG. 1. HAV detection sensitivity of SPRIA, ELISA, and A-ELISA. Twofold dilutions of purified HAV (initial concentration, 4×10^7 particles per ml) were tested to determine the minimal number of virus particles required to give a positive result (P/N = 2.1, [------]) in ELISA (○), SPRIA (●), or A-ELISA (▲). P/N, Positive-to-negative ratio.

sensitivity was obtained when alkaline phosphatase-conjugated streptavidin, rather than alkaline phosphatase avidin-D, was used. Linking biotinylated MAb and biotinylated alkaline phosphatase with avidin-D failed to increase test sensitivity.

The sensitivity of the A-ELISA was compared with those of the SPRIA and the standard ELISA in tests performed in triplicate on samples of twofold dilutions of CsCl-purified HAV (Fig. 1). In the A-ELISA, a minimum of 2×10^5 particles per ml was required for a positive result. The results indicate that the A-ELISA was approximately five times more sensitive than the standard ELISA and two times more sensitive than the SPRIA.

Since detection of the low numbers of enteric viruses believed to occur in environmental samples requires some type of concentration step, interference may result from the eluents used or natural substances concentrated along with virus particles. The proteinaceous solutions used for elution and organic substances, such as humic and tannic acids, potentially present in surface waters were tested for their effect on A-ELISA sensitivity. No significant loss of test sensitivity was observed with any of the eluents tested or with various dilutions of humic and tannic acids. The latter is especially significant since the concentrations of humic and tannic acids used were 20-fold greater than the concentrations reported for surface waters.

Since it is possible for enteric viruses other than HAV to be present in polluted waters, it was important to determine if such viruses would cross-react in the A-ELISA, causing false-positive tests. Poliovirus 1, coxsackievirus B2, echovirus 7, and the WA strain of rotavirus were tested to establish the specificity of the test. The results are shown in Table 1. No significant differences were observed between the values obtained with the negative control (0.01 M PBS) and those obtained with the viruses tested.

From April to October 1986, 20 estuarine samples were collected and processed. The samples were tested for HAV antigen by A-ELISA and SPRIA. HAV was detected in 3 of 20 (15%) water samples by A-ELISA, whereas only 1 sample of 20 (5%) was found positive by SPRIA. The positive results obtained were confirmed by competitive A-ELISA and SPRIA. Human IgG PAb blocked the specific binding of

TABLE 1. Evaluation of specificity of A-ELISA[a]

Samples tested	OD_{414}[b]	P/N[c]
Poliovirus 1	0.04	1
Echovirus 7	0.04	1
Coxsackievirus B2	0.02	0.5
Rotavirus WA	0.03	0.75
HAV	0.76	19.0
0.01 M PBS	0.04	

[a] Three enteroviruses and the WA strain of rotavirus (10^5 to 10^6 PFU/ml) were used as antigen in the A-ELISA. Purified HAV and 0.01 M PBS (pH 7.2) were used as positive and negative controls, respectively.
[b] OD_{414}, Optical density at 414 nm.
[c] P/N, Positive-to-negative ratio.

biotinylated IgG MAb and ^{125}I-labeled human IgG MAb to HAV.

The development of an A-ELISA for detecting HAV in estuarine water samples represents an adaptation of ELISA methods to detection of waterborne HAV. The enhanced sensitivity of the A-ELISA makes it a valuable tool for monitoring the HAV pollution of surface waters. The A-ELISA was approximately 50 times less sensitive than either radioimmunofocus or immunofluorescence tests on the basis of electron microscope counts of the minimal number of physical particles of HAV needed for a positive result in each test. However, in both of these assays, the virus must replicate in cultured cells, and since wild strains of HAV replicate slowly, if at all, in vitro, the radioimmunofocus and immunofluorescence tests are of little value in the analysis of environmental samples.

To detect HAV contamination, water samples had to be concentrated up to 1,000-fold before analysis. It was important to determine whether some naturally occurring substances were concentrated along with the virus or some protein constituents of eluents used for virus recovery affected test sensitivity. Our results show that neither humic or tannic acid nor any of the eluents tested had any influence on the A-ELISA. The specificity of the test was attributed to the use of MAb with a high reactive affinity. Negative test results obtained with enteroviruses and rotavirus indicated that screening tests for HAV could be carried out with little or no interference from other enteric viruses that might be present.

Although the transmission routes of HAV have been well described in epidemiological studies, the virus has not been found routinely in water because of the lack of a method for its direct detection. Consequently, little is known about the prevalence of HAV in surface and groundwaters. HAV antigen was detected by RIA in one groundwater and two sewage samples in Georgetown, Tex. (8). In the present study, HAV antigen was detected in 3 of 20 estuarine water samples by A-ELISA, while only 1 of these 3 was positive by RIA. These results are in agreement with the reported sensitivities of A-ELISAs developed for Norwalk (7) and herpes simplex (1) viruses. Although the number of field samples included in this study was small, the results indicate that A-ELISA can be used to monitor HAV pollution of estuarine waters. Detection of HAV in waters used for recreation and shellfish harvesting could help to reduce or prevent the incidence of outbreaks resulting from HAV transmission via these routes.

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