TWO COMPETITIVE ENZYME IMMUNOASSAYS FOR THE DETECTION OF IgG CLASS ANTIBODIES TO HEPATITIS A ANTIGEN

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Two competitive enzyme immunoassays (EIA) techniques were developed: in the first (COMP-1), test sera were added together with HAV antigen on anti-HAV IgG-coated wells followed by an anti-HAV HRP conjugate; in the second (COMP-2), test sera and anti-HAV HRP conjugate competed for HAV epitopes previously adsorbed to anti-HAV IgG-coated wells. Both procedures used tetramethylbenzidine (TMB) as a substrate. Both competitive tests were shown to be reproducible and suitable for routine diagnosis and research purposes.

Key-words: Enzyme immunoassay. Anti-HAV. Hepatitis A.

Epidemiological observations have demonstrated that hepatitis A virus (HAV) occurs throughout the world. In our country, is responsible for 50% of hepatitis cases diagnosed at the National Reference Center for Viral Hepatitis, Oswaldo Cruz Foundation, Rio de Janeiro.

Following the discovery of hepatitis A virus (HAV)[6], a number of methods such as immuneadherence haemagglutination[15] and complement fixation[17] have been described for the detection of anti-HAV antibodies and for the separation of early (IgM) and late (IgG) antibodies[12]. More recently, third generation tests such as radioimmunoassay[9 20], and enzyme linked immunosorbent assay[4] have been developed and shown to be suitable for routine serodiagnosis.

The propagation of HAV in various cells lines[5 7 10 11 14 18], has provided an alternative to the use of feces collected in the preicteric phase from human hepatitis A cases or from experimentally infected primates as sources of antigen for serological assays.

This paper describes the development and standardization of two competitive enzyme immunoassays (EIA) for HAV antibodies and compares the results with those obtained with a commercial EIA.

MATERIAL AND METHODS

Sera

Standardization of both competitive tests was performed using convalescent serum from a patient with serologically confirmed hepatitis A as positive control and an antibody-negative serum as negative control. The reproducibility of both procedures was evaluated using a reference panel with twenty-two sera previously tested for anti-HAV antibodies by a commercial EIA kit (Hepanostika anti-HAV, Organon Teknika, Holland). The panel had twelve sera serologically diagnosed as hepatitis A and ten sera from healthy blood donors without anti-HAV antibodies.

Antigen Preparation

Two strains of HAV virus wére grown in fetal rhesus monkey kidney cells (FRhK-4), HAS-15 (Center for Disease Control) and HM-175 (Walter Reed Army Institute). Five ml of each virus (O.D. at 450 nm = 0.235 in EIA HAV-TEST – in house production) was adsorbed at 37°C onto confluent cell sheets of 150cm² tissue culture flasks (Falcon, Becton Dickinson Co) for two hours. After adsorption, cultures were maintained at 37°C with 50ml of Williama's medium (Sigma Chemical Co) supplemented with 2% L glutamin 0,83 ml (Sigma), 10mM MEM Non Essential Amino Acids 0,83 ml (Sigma), 1M HEPES 1.66 ML (Sigma), 7.5% sodium bicarbonate 1.66 ml, 50 mg/ml gentamicin 0.25 ml and 2% fetal calf serum (Sigma). At weekly intervals, tissue culture fluid (TCF) of each bottle was harvested for HAV antigen titration (EIS HAV-TEST) and replaced with fresh medium. Maximum release of antigen ocurred 21 days post-infectio (p.i.) in cells inoculated with HAS-15 and 28 days p.i. with HM-175. TCFs were pooled and concentrated with 10% Polyethylene glycol (Sigma) to give aproximately O.D. 1.00 (450 nm) in ELISA HAV-TEST.

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The antigen was divided in aliquots of 200 μl sufficient for one microplate strip to avoid repeated freeze-thawing and stored at – 70 C.

Antiserum preparation

Convalescent serum was obtained from a patient with serologically confirmed hepatitis A with a anti-HAV titre of 1/200,000 in EIA (Hepanostika anti-HAV, Organon). Immunoglobulin G was purified from 10,0 ml of the serum by 2 step precipitation with ammonium sulfate, giving final concentrations 55% and 33% respectively, followed by ion-exchange chromatography on DEAE-celulose (DE-52, Whatman, as described by Purcell et al[19]. Purified IgG was stored at –20ºC and used for antibody capture in a solid phase and for the preparation of enzyme linked antibody.

Anti-HAV solid phase preparation

Purified anti-HAV lgB with a protein concentration of 16.8 mg/ml was diluted 1/10 in PBS (pH 7.2)/Glycerol 50% and stored in aliquots of 2,0 at –20ºC until used. Activity was assayed by serial dilutions of this antibody preparation.

Conjugate Preparation

Human anti-HAV IgG (4 mg/ml) was conjugated to 10 mg/ml horseradish peroxidase (type VI; Sigma) by Nakane's method modified by Camargo et al[2].

Checkerboard Titration

In both tests, the wells of polyvinyl microplates (Hemobag Produtos Cirurgicos Ltda) were coated with 100 μl of anti-HAV IgG diluted in 50 mM carbonate buffer pH 9.6 (1.51g Na_2CO_3, 3.00g $NaHCO_3$ in 1L of distilled water), covered and left overnight at 4ºC. The first vertical strip was left empty in all following steps of the reaction, except at the end of the reaction, when this strip was used for substrate control and blank reading. After incubation, the wells were emptied and washed four times with phosphate buffered saline pH 7.2 (PBS) containing 0.5% of bovine serum albumim (Sigma).

Competitive Test 1 (COMP-1)

In this procedure, after the washing step, 50 μl of serial serum dilutions in PBS pH 7,2 were added into each well. Positive and negative sera were used as controls. Next, an aliquot of 50 μl of HAV antigen diluted in PBS pH 7.2 was dispensed into the wells.

The microplates were incubated at 37ºC for 1 hour, washed four times with PBS pH 7.2 containing 0.05% of Tween 20 (PBS T). A volume of 100 μl of the human anti-HAV IgG conjugate diluted in PBS pH 7.2 with 10% of normal human serum and 10% of normal goat serum was added into each of the wells and the plates were incubated at 37ºC for 1 hour. After washing four times with PBS T, 150 μl of substrate tetramethylbenzidine (TMB, Sigma) 10 mg/ml DMSO (Sigma) was added into the wells in the proportion of 6 ml phosphate-citrate buffer pH 5.0 (sodium acetate 34 g, citric acid 0,73 g in 250 ml of distilled water), 65.5 μl TMB and 7.2 μl H_2O_2 30%. *After 10 minutes, the reaction was stopped by adding* 50 μl of H_2SO_4 2M. The plates were read photometrically at 450 nm.

Competitive Test 2 (COMP-2)

After the initial washing step, 100 μl of HAV antigen diluted in PBS pH 7.2 was dispensed into the wells and the plates were left 1 hour at 37ºC for antigen adsorption. The plates were washed four times with PBS T and serum specimens (50 μl) were added into the wells, followed by 50 μl of conjugate diluted in PBS pH 7.2 with 10% normal human serum and 10% normal goat serum and incubation at 37ºC for 1 hour. After washing four times with PBS T, 150 μl of TMB substrate was added and the reaction was stopped and read in the same way as describe in COMP-1.

Determination of Cut-off Value

Cut-off levels of both procedures were calculated by dividing the sum of the negative control mean absorbance and the positive control mean absorbance by 2. Specimens with absorbance values greater than than the cut-off value were negative and those with absorbance values lower than or equal to the cut-off value were considered positive, corresponding to 50% of inhibition. Typical negative specimens gave an optical density between 0.60 and 0.80.

RESULTS

Three checkerboard titrations were done to determine the opimal dilutions of the reagents for each competitive test. The results were expressed as N/P ratio.

Competitive Test 1 (COMP-1)

Figure 1a shows the checkerboard titration between serum specimen and capture antibody. The capture antibody was diluted from 1/1000 to 1/32000. Positive and negative sera were tested undiluted and

diluted 1/10 and 1/30. Conjugate antibody and HAV antigen were assayed without variation (1/1000 and 1/2, respectively). Three curves were obtained and the peak observed in each reveals the optimal concentration of the reagents tested. It can be seen that antibody capture diluted 1/8000 and sera diluted 1/10 gave better results (O.D. 0.860) although greater N/P values were found in more diluted reagents.

Figure 1b shows the checkerboard titration between capture and conjugate antibody. Capture antibody was diluted from 1/1000 to 1/8000 and three dilutions of the conjugate antibody, 1/500, 1/1000 and 1/2000, were assayed. Positive and negative sera and HAV antigen were used in constant dilutions, 1/10 and 1/2 respectively. Conjugate antibody diluted 1/1000 gave satisfactory results with capture antibody diluted 1/8000 (O.D. 0.868) when compared with those obtained before (Figure 1).
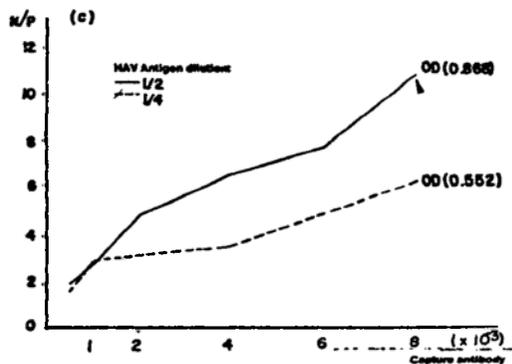

Figure 1 – COMPO-1 checkerboard titrations (°)
 (a) Checkerboard titration between serum specimens and capture antibody
 (b) Checkerboard titration between conjugate and capture antibody.
 (c) Checkerboard titration between HAV antigen and capture antibody
 (°)
 (*) Values in parenthesis represent the optical density obtained with the negative controls. Arrows indicate the optimal dilutions of each of the reagents.

Figure 1c displays the checkerboard titration between the HAV antigen diluted 1/2 and 1/4 and capture antibody, diluted in the same way as described at Figure 1b. Positive and negative sera and conjugate antibody were assayed in constant dilutions, 1/10 and 1/1000 respectively. Better results were achieved using more concentrated HAV antigen (1/2) and more diluted capture antibody (1/8000).

The optimal reagent concentrations found on COMP-1 were capture antibody 1/8000, HAV antigen 1/2, test sera 1/10 and conjugate antibody 1/1000.

Competitive Test 2 (COMP-2)

For the competitive test 2, three checkerboard titrations were also made. Firstly, the concentrations of the components that compete on assay, sera and conjugate antibody, were tested (Table 1). Conjugate was used in two dilutions (1/500, 1/1000) and sera were tested undiluted and diluted 1/10. Better results were obtained when more concentrated components were used (N/P 13.2). Background color, caused by free HAV-specific antigenic sites, appear if sera are used diluted 1/10 (see optical density of the positive controls).

For the checkerboard titration of capture and conjugate antibody, five and two dilutions were tested respectively as shown in Figure 2a. Positive and negative sera were assayed undiluted and HAV antigen was diluted 1/2. Higher N values were obtained at
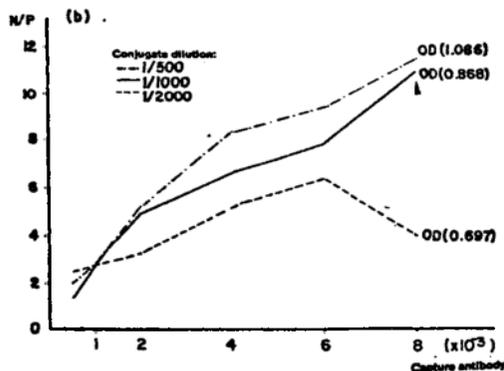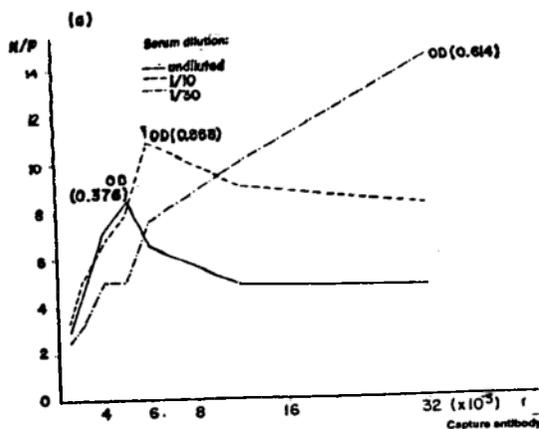
Table 1 – *Checkerboard titration between controls sera and conjugate antibody (COMP-2). Results were expressed in optical density (450 nm).*

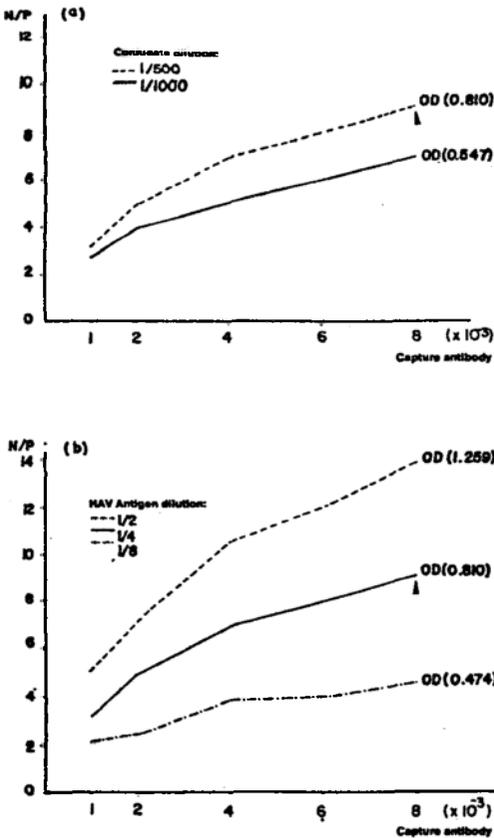| Conjugate antibody	Controls sera Undiluted		N/P	1/10		N/P
	positive	negative		positive	negative	
1/500	0.077	1.014	13.2	0.245	1.330	5.4
1/1000	0.054	0.663	12.3	0.154	0.952	6.2

Figure 2 – *COMP-2 checkerboard titrations (*)*

(a) Checkerboard titration between conjugate and capture antibody

(b) Checkerboard titration between HAV antigen and capture antibody

(*) Values in parenthesis represent the optical density obtained with the negative controls. Arrows indicate the optical dilutions of each of the reagents.

1/500 dilution of conjugate and 1/1000 dilution of capture antibody.

Checkerboard titration of HAV antigen and capture antibody is shown in Figure 2b. Three dilutions of the virus were tested (1/2, 1/4, 1/8); capture antibody was diluted as described in Figure 2a. Positive and negative sera were assayed undiluted and conjugate antibody was diluted 1/500. According to N/P results, HAV antigen can be used diluted either 1/2 or 1/4, but capture antibody must be diluted 1/8000.

The optimal reagent concentrations found on COMP-2 were capture antibody 1/8000, HAV antigen (1/4), test sera undiluted and conjugate antibody 1/500.

A reference panel (ELISA-ORGANON) with 12 hepatitis A positive sera and 10 hepatitis A negative sera was utilized to evaluate the reproducibility of both competitive procedures. There was agreement between ELISA-Organon, COMP-1 and COMP-2 in all sera tested. The results of both competitive assays are shown at Figure 3. O.D. values of positive specimens ranged from 0.052 to 0.094 on COMP-1 and 0.047 to 0.069 on COMP-2, whereas those of negative specimens ranged from 0.283 to 0.449 on COMP-1 and 0.246 to 0.396 on COMP-2.

DISCUSSION

The main factor that hampered the standardization of both competitive ELISA was the growth of HAV virus and the obtention of HAV antigen in sufficient amounts that could be employed as antigen on both tests. We used two standard strains of HAV: HAS-15, which was originally isolated by the Center for Disease Control, Atlanta, GA., and HM-175, that was isolated in Australia[13]. Rakela et al[21], had shown the morphological and serological similarities between two geographically diverse strains (Los Angeles, USA and Rosario, Argentina) and these findings supported the use of non-Brazilian strains on our study, although we have now sucessfully adapted a Brazilian isolate. Having established a culture system in FRhK-4 cells[8 23], it was found that maximum release of antigen in the TCF was on day 21 for HAS-15 and on day 28 for HM-175. The culture system was optimized to yield, after approximately 20X concentration with PEG, enough antigen for 100 reactions from each 150
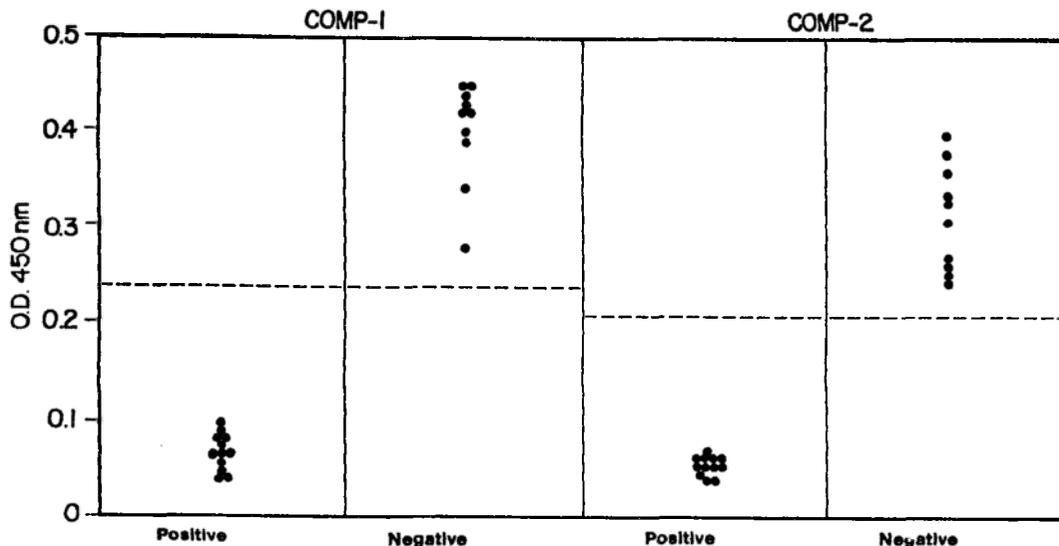
Figure 3 – *Competitive IgG anti HAV EIA (COMP 1, COMP 2) of panel sera. Dashed corresponds to the cut-off for each of the tests.*

cm^2 culture bottle. As shown by Flehming et al[9], both stool-derived HAV as cell-culture produced HAV react identically in tests for anti-HAV antibodies in human sera. The use of cell-culture derived HAV es diagnostic reagent free of the inherent problems associated with reagents of human or primate origin[4].

The object of the present study was to standardise and compare two EIA competitive methods for the detection of anti-HAV antibodies, with the aim of developing an assay giving clear cut results and being easy to perform and economic in reagents.

Four components of both procedures (COMP-1, COMP-2) were analysed: capture and conjugate antibody, serum specimens and HAV antigen. The choice of an optimal dilution of reagents in each checkerboard titration made possible both N/P value and visual discrimination between positive and negative samples. Positive samples must give O.D. (450 nm) smaller than 0.100 and negative ones higher than 0.700. Given the high costs in producing conjugates and the difficulty in obtaining HAV antigen, the quantity needed of these reagents was also an important consideration.

Like Parry[16], we also found that the most critical procedure to improve sensitivity was to determine the coating antibody dilution that gives the greatest discrimination between negative and positive samples (N/P). In all checkerboard tests made for both assays, it was seen that dilution of capture antibody increase N/P ratios whatever the component being tested. A 2- to 4- fold deviation of the optimal dilution

may result in 55.5% loss of sensitibity on COMP-1 and 46,15% on COMP-2. In COMP-1, the competition occurs between capture and serum specimen antibodies by the HAV antigen epitopes; hence, sera dilution will depend upon the dilution of the capture antibody used.

In competitive assays, it is important to limit the amount of antigen to provide the greatest sensitivity. Studies have shown that too much antigen requires a lot of antibody activity in the specimen to adequately block the antigenic sites. Concerning HAV antigen, better results were achieved using lower dilutions, which showed no prozone effect[16]. However, as its production is a critical factor, the dilution used on both procedures was the minimum that acceptable N/P ratio. We also observed that repeated freeze-thawing of the HAV antigen can greatly reduce its titre and consequently, the sensitivity of the test. Is was observed when we evaluated both procedures using a reference panel. It is recomended that HAV antigen be stored in small aliquots and always thawed in an ice bath.

In COMP-2, as the competition occurs between serum specimen and conjugate antibodies, sera cannot be diluted, otherwise free sites on HAV antigen will permit binding of conjugate antibodies producing background color in positive samples and consequently, false positive results.

Satisfactory results were achieved using more diluted conjugate antibody (1/1000) in COMP-1 in spite of greater N/P value obtained at dilution 1/500.

In this case, is preferable to choose a dilution with lower N/P value but with no background color on positive sera samples to prevent false nagative results. The color of negative samples in COMP-2 test is proportional to the amount of conjugate antibody reacted. Hence, the relation between serum specimen and conjugate antibodies must be one that gives no color with positive sera, and higher O.D. with negative sera.

Horseradish peroxidase can be detected with a variety of substrates which usually become oxidized to an insoluble polymer when the peroxidase catalyses the release of oxygen from hydrogen peroxide. We chose TMB as substrate after tests with o-phenylenediamine (OPD). Basides being safer, TMB was shown to be more sensitive, as also observed by Bos et al[1].

The time spent in the performance of the test was also considered important in Hepatitis A diagnosis. After an overnight incubation with capture antibody, we reduced to approximately 3 hours the period of both procedures, which is shorter than those of other competitive immunossays[3] [22].

We conclude that both competitive tests are suitable for diagnostic routine, with high sensitivity and specificity. Moreover, they can be easily reproduced and applied in any diagnostic laboratory besides being useful as an important tool in Hepatitis A research.

RESUMO

Foram desenvolvidos dois ensaios imunoenzimáticos (EIA) competitivos: no primeiro (COMP-1) colocou-se numa placa sensibilizada com anti-HAV IgG as amostras teste juntamente com o antígeno HAV e a seguir o conjugado anti-HAV HRP; no segundo (COMP-2), as amostras teste e o conjugado anti-HAV HRP competem pelos epitopos do antígeno HAV previamente absorvido na placa sensibilizada do anti-HAV IgG. O substrato utilizado foi tetrametilbenzidina (TMB). Ambas as técnicas mostraram ser produtíveis e aplicáveis para fins de diagnóstico e pesquisa.

Palavras-chaves: Enzyme immmunoassay. Anti-HAV. Hepatitis A.

ACKNOWLEDGMENTS

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