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In situ enzyme immunoassay for titration of a Brazilian hepatitis A virus strain (HAF-203)

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Abstract

Hepatitis A virus (HAV) replicates relatively slowly in cell culture without a cytopathic effect, a fact that limits the use of tissue culture assays. The radioimmunofocus assay is the standard method for HAV titration, although it is labor intensive and requires the use of radioisotopes. A simple, rapid and objective infectivity assay based on an *in situ* enzyme immunoassay (EIA) is described here for a Brazilian cell culture-adapted HAV strain (HAF-203). The assay uses a peroxidase-labeled polyclonal antibody to fixed monolayers as an indicator of infection. EIA may be completed within 7 days using serial 5-fold dilutions of the virus, yielding a titer of 5.024 log 50% tissue culture infective dose ($TCID_{50}$)/ml for HAF-203. This technique had a detection limit of 1.1 log $TCID_{50}$/ml and the specificity was demonstrated by detecting no reaction on the columns of uninfected wells. The reproducibility (with intra- and inter-assay coefficients of variation ranging from 1.9 to 3.8% and from 3.5 to 9.9%, respectively) and quantitation of the assay were demonstrated by close agreement in virus infectivity titers among different assays of the same amount of virus and between assays of different amounts of virus. Furthermore, this assay does not require the use of radiolabeled antibodies. We describe here an efficient EIA that is highly reproducible and that could be used to monitor HAV growth in cell culture and to determine the quantity of HAV antigen needed for diagnostic assays. This is the first report of the infectious titer of the Brazilian cell culture-adapted HAV strain (HAF-203).

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Hepatitis A virus (HAV) is a representative member of the hepatovirus genus within the family Picornaviridae (1). The 27-nm particle is non-enveloped and contains a single strand of positive-sense RNA as the genome (2). Since it was first adapted to cell culture by Provost and Hilleman (3), HAV has replicated slowly in most reported systems and establishes persistent infections in tissue cultures (3,4), although faster growing strains of HAV have been isolated (5). A number of different cell types are susceptible to HAV infection (6) but fetal Rhesus monkey kidney cells proved to be one of the most susceptible cell line for antigen production (7).

The slow, non-cytopathic growth of HAV

has hampered its study in tissue culture and its quantitation. Prior to adaptation of the virus to growth in tissue culture, HAV antigens were quantitated by radioimmunoassays (RIA) (8). Subsequent methods developed to assay HAV in tissue culture include radioimmunofocus assay (RIFA) (9), *in situ* RIA and fluorescent focus assay (10). All of these assays are complex and time consuming. Furthermore, all three procedures have been commonly used to monitor the production of HAV antigen as an indirect parameter of viral growth (3,8,10,11). While few assay methods have been developed for HAV titration (11-14), there is still a need for the development of technically simpler, more rapid, reproducible, and quantitative *in vitro* assay procedures.

HAV infection still represents an important public health problem in Brazil. HAV was responsible for more than 50% of the acute cases of hepatitis diagnosed at the National Reference Center for Viral Hepatitis in Rio de Janeiro during 1999 and 2001 (15) but the real incidence of infection is underestimated because of underreporting and of asymptomatic cases that are very common among children and adolescents. Although hepatitis A is endemic in Brazil, only one successful propagation of a Brazilian HAV strain (HAF-203) has been reported (16). HAF-203 propagates in fetal Rhesus kidney (FRhK-4) cells within 7 days post-incubation without a cytopathic effect and has proved to be suitable for antigen production. In the present study, we describe a simple, rapid and reproducible infectivity assay based on an *in situ* enzyme immunoassay (EIA) for the fast-growing cell culture-adapted HAV strain (HAF-203).

A continuous FRhK-4 cell line was purchased from the Adolfo Lutz Institute at passage number 92. Cell cultures were grown at 37ºC in 175-cm² disposable Falcon flasks containing William's medium E supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 0.1 mM MEM non-essential amino acids, 25 mM HEPES, 0.18% sodium bicarbonate, 100 U/ml penicillin, and 100 µg/ml streptomycin. All chemicals, media and drugs were obtained from Sigma Chemical Co.

HAV strain HAF-203 was isolated from FRhK-4 cells of an acute phase human fecal specimen. The isolation and passage history of HAV strain HAF-203 have been described previously (16). In an attempt to select a rapidly growing virus population, the seventh passage of HAV strain HAF-203 was performed more than 20 times at 7-day intervals. Briefly, FRhK-4 cells were grown to confluence in disposable 150-cm² Falcon flasks. Monolayers were washed once with 0.01 M phosphate-buffered saline (PBS), pH 7.2, and inoculated with 3.0 ml HAV strain HAF-203. The virus was adsorbed for 1 h at 37ºC, and 40 ml of maintenance medium was then added without removal of the inoculum. Cultures were kept at 37ºC for 7 days. Cell-associated virus was extracted by freeze-thawing the bottle contents, including the medium supernatant, three times. The harvested fluid was subjected to a short burst of sonication followed by a brief low-speed centrifugation and the supernatant was aliquoted and stored at -70ºC.

Flat-bottomed 96-well microtiter plates (tissue culture grade Nunc Inc., Naperville, IL, USA) were seeded with 100 µl of a suspension of FRhK-4 cells prepared in growth medium ($\cong 10^{4.5}$ cells/ml). After incubation at 37ºC in a 5% CO_2 atmosphere for 24 h until confluence was just achieved, 100-µl dilutions of virus sample (prepared in William's medium E supplemented with 2% heat-inactivated fetal bovine serum) were added to test eight wells per dilution without removal of growth medium. To ensure detection of an endpoint, 3-fold (5- and 10-fold) serial dilutions of each sample were prepared to inoculate two microtiter plates (i.e., a total of 60 dilutions). Two columns of uninfected wells were included on each plate, as well as one extra column on the first plate for blanking the plate reader. Monolayers

were then incubated for 5, 7, 14, 21, 28, and 35 days, after which the medium was removed. Cells were fixed using 100 µl per well of a 1:1 mixture of acetone and methanol (precooled to 4ºC) for 5 min. The fixative was removed and monolayers were allowed to dry uncovered at room temperature for at least 2 h.

A pool of convalescent serum was obtained from patients with serologically confirmed hepatitis A with an anti-HAV titer of ≥1:100,000 in EIA (Hepanostika anti-HAV, Organon) 100 days after the onset of the disease. Immunoglobulin G was purified from 10 ml of this serum by 2-step precipitation with saturated ammonium sulfate, giving final concentrations of 55 and 33%, respectively, followed by ion-exchange chromatography on DEAE-cellulose (DE-52, Whatman), as described by Purcell et al. (17). Human anti-HAV IgG (4 mg/ml) was conjugated to 8 mg/ml horseradish peroxidase (type VI; Sigma) by the method of Nakane modified by Camargo et al. (18). The resulting conjugate was stored at -20ºC in PBS/glycerol (v/v) and could be used to titrate other HAV strains since these isolates had antigenic relatedness, as shown by Lemon and Binn (11). The presence of virus-specific antigen was detected using the conjugate diluted at 1:8000 with PBS/0.5% bovine albumin (w/v). The conjugate (100 µl) was added to each test and uninfected well, but not to blank wells. After incubation at 37ºC for 1 h, the antibody solution was removed and monolayers were washed four times (at 400 µl/well) with PBS plus 0.1% Tween 20 (w/v) using an automated plate washer (Organon Teknika, Netherlands). Immediately after washing, bound antibody was detected using hydrogen peroxide and tetramethylbenzidine. The color change was monitored visually and stopped by the addition of 100 µl 1 M sulfuric acid per well after approximately 15 min. The absorbance of the plates was then read at 450 nm and a positive/negative (P/N) ratio was determined using uninfected wells as a negative value.

Wells were easily identified by the unaided eye as either positive or negative, with no intermediate results being noted. The titer of infectious virus was determined by the method of Reed and Muench (19) which is based on the 50% tissue culture infective dose ($TCID_{50}$) endpoint. Using the assay format described, it was possible to determine infectious virus titer at the 95% confidence level, with the unit being $TCID_{50}$/ml of undiluted sample.

Each two plates of cells were inoculated with serial dilutions of HAV (3-, 5- and 10-fold) and incubated for varying periods of time (5, 7, 14, 21, 28, and 35 days) before fixation and staining with the antibody. Absorbance at 450 nm for each dilution was averaged from 8 wells. Figure 1 shows that the highest EIA values were obtained after incubation for 14 days, with no benefit from a longer incubation being noted.

The $TCID_{50}$ was determined after opti-

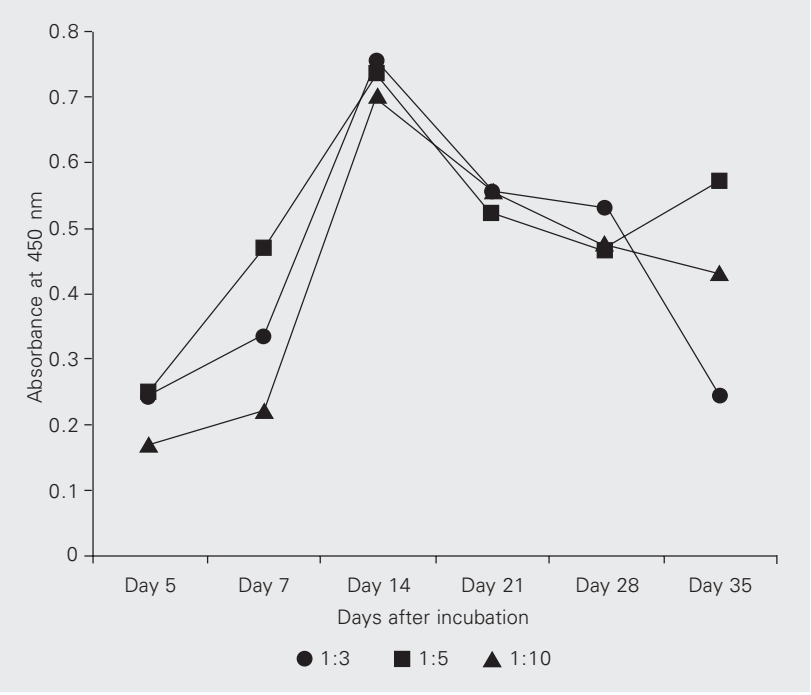

Figure 1. Assay of hepatitis A virus strain HAF-203. Eight wells were used for each dilution (1:3; 1:5; 1:10) for each day post-incubation. The initial dilutions are given in the inset. Data are reported as means of absorbance at 450 nm. SD was 0.01-0.16.

mization of the conditions, including the number of cells. The optimum number of FRhK-4 cells was found to be ~$10^{4.5}$ cells/well. If more than $10^{4.5}$ cells were added to each well, the cells quickly overgrew and began to die, and with less than 10^4 cells/well, the staining of negative control wells was less distinct. Using the optimized conditions, we determined the titer of the tissue culture-adapted strain of HAV (HAF-203) after different periods of incubation and different virus dilutions. We observed that the highest $TCID_{50}$ value occurred on day 7 using serial 5-fold dilutions and therefore we used these conditions for subsequent assays. The log $TCID_{50}$ determined by the method of Reed and Muench (19) was $10^{-4.024}$. Since only 100 µl of diluted virus was placed in each well, this figure was adjusted by a factor of ten to give a titer of 5.024 log $TCID_{50}$/ml.

To test the robustness of the assay, the virus infectivity titer of one-virus aliquot was determined ten times using cell cultures prepared from a similar batch of cells. The intra-assay variability was 1.1-fold in $TCID_{50}$ titers. Virus titers from ten different aliquots of the same virus showed an average inter-assay variation of 1.2-fold.

A simple and reproducible assay system for HAV is in increasing demand. The need for such an assay is particularly important to validate virus inactivation, to follow up HAV growth in cell cultures and to establish the quantity of antigen for diagnostic assays. For these studies, the detection of antigen and/or nucleic acid is not appropriate since these constituents considered separately are either noninfectious (capsid protein), or of greatly reduced infectivity (naked, full-length, genomic RNA). RIFA, the gold standard assay, is extremely labor intensive and has the disadvantage of relying on radiolabeled antibody detection. The present study indicates that this *in situ* EIA is a practical and accurate procedure for the determination of HAV titer.

A number of HAV infectivity assays have been performed, the most basic ones using plaque formation as an indicator of infection by a single virus (20). However, cytopathogenic effect is an indirect indicator of infection and when titrating infectious virus under potentially cytotoxic conditions, the environmental effect on cells can be mistakenly interpreted as virus-induced cytopathogenic effect. Furthermore, many HAV strains have no cytopathic effect, a fact that makes it difficult to use this method for their titration.

The limit of detection for the assay described in the present study was 1.1 log $TCID_{50}$/ml. This limit is lower than that previously reported by Borovec and Uren (13) and Yap and Lam (20). The small variations in titers observed in intra-assays and inter-assays (with coefficients of variation ranging from 1.9 to 3.8% and from 3.5 to 9.9%, respectively) demonstrated the reproducibility and accuracy of the EIA. Since EIA requires no microscopic examination of the wells, it is faster and less tedious, especially when many samples are involved. Another advantage of EIA is that peroxidase staining is permanent and the material can be stored for a long time. Also, EIA can be performed on cells grown in plastic wells, whereas other techniques such as immunofluorescence must be applied to cells grown on either glass coverslips or slides. In addition to being simpler and easier to perform, the EIA procedure is safer than the RIFA developed by Lemon et al. (9). The time required for this assay, with the entire procedure from fixation to examination of 96 coverslips requiring 3 to 4 h, is much shorter than the time needed for similar assays using radioactive markers (9).

Hepatitis A virus is known to grow slowly and to low titers in most cell culture systems (3,4), a property that hampers its mass production *in vitro* for vaccines or diagnostic tests. Efficient HAV replication as described for HAF-203 (5) provides useful material for HAV diagnostic assays. Thus, a method for HAV titration in cell culture is

important to monitor HAV growth in these systems and to determine the quantity of antigen for diagnostic assays. The present report describes an efficient EIA for these objectives and reports the titration of a Brazilian strain of HAV.

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