An immunoenzymatic assay for the diagnosis of hepatitis A utilising immunoglobulin Y

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The detection of anti-hepatitis A virus (HAV) antibody levels by diagnostic kits in the convalescent period of disease generally use immunoglobulin G (IgG), which is expensive. An alternative to IgG is immunoglobulin Y (IgY), an immunoglobulin antibody encountered in birds and reptiles. The aim of this study was to develop a competitive immunoenzymatic assay to measure total anti-HAV antibody levels using anti-HAV IgY as the capture and conjugated immunoglobulins. For this purpose, anti-HAV IgY was conjugated to horseradish peroxidase (HRP) and the optimal dilution of HRP-conjugated antibodies was evaluated to establish the competitive immuneenzymatic assay. The results obtained from our "in-house" assay were plotted on a receiver operator curve, which showed a sensitivity of 95% and a specificity of 98.8%, demonstrating that a competitive anti-HAV IgY immunoenzymatic assay developed "in house" could be used as an alternative to commercial assays that utilise IgG.

Key words: IgY - hepatitis A - ELISA

Hepatitis A virus (HAV) is a non-enveloped RNA virus that belongs to the Picornaviridae family and the Hepatovirus genus. Usually, natural infection occurs from the ingestion of contaminated faeces, food or water or from person-to-person contact (Wheeler et al. 2005). Acute hepatitis infection is endemic in developing countries, where 1.4 million new infections occur each year (Saha et al. 2009). In these countries, the epidemiological profile of HAV is changing from high to intermediate endemicity. The changing to an intermediate endemicity led to growing numbers of susceptible young people, which leads to increased potential for outbreaks (Villar et al. 2002). Improvements in sanitation and awareness programmes in countries with intermediate endemicity have reduced early childhood exposure to HAV. However, because the virus has not been eliminated from the environment, there is an increase in the number of susceptible people who are of working age, which negatively impacts public health and the economy. Besides that, these people are more likely to suffer harsh symptoms of the disease, which leads to fulminant hepatitis in 0.14% of cases (Leung et al. 2005). The universal vaccination and screening of susceptible people in highly endemic countries have been effective at preventing and curbing outbreaks, but this effort has been limited by the cost of the vaccine (Costas et al. 2009). Purified human anti-HAV immunoglobulin G (IgG) has been used as the key component of diagnostic assays and is recommended for as post-exposure prophylaxis. However, the production of this immunoglobulin has been limited and is expensive (Victor et al. 2007).

An alternative to using IgG in prophylaxis and immunodiagnostics is to use immunoglobulin Y (IgY). IgY is present in egg yolk and in the serum of birds and reptiles. The phylogenetic distance between mammals and birds gives to IgY certain advantages: an increase in the humoral immune response against mammalian proteins (Carlander et al. 2002), complement system is not activated by IgY (Schade et al. 2005) and no cross reactivity with IgG (Kummer & Li-Chan 1998). Additionally, the amount of IgY produced in egg yolk is threefold greater than the amount of IgG in the same volume of rabbit serum (Cova 2005), the production expense is less than IgG (Cova 2005), IgY presents a reduction in background colour of immunoenzymatic assays when compared to IgG (Schade et al. 2005) and the extraction of IgY is simpler than IgG (can be made merely by egg yolk collection instead of animals bleeding) (Cova 2005). Because of these advantages, IgY has been successfully used in immunoenzymatic assays for the detection of *Schistosoma japonicum* (Lei et al. 2009), foot-and-mouth disease virus (Veerasami et al. 2008), sendai virus in porcines (Kvietkauskaite & Acaite 2004) and *Escherichia coli* (Sunwoo et al. 2006).

Considering the epidemiological change in hepatitis A disease in developing countries around the world and the rise in the morbidity of the disease, it would be of interest to develop alternative methods for the diagnosis of hepatitis A. In this study, we developed a new competitive immunoenzymatic assay that can detect specific antibodies against HAV by utilising IgY obtained from chickens immunised with HAV antigen.

Initially, HAV strain HAF-203 was propagated in FRhK-4 cells (Rhesus monkey foetal kidney cell line) for 14 days, aliquoted and stored at -70ºC. The hepati-

Financial support: CNPq (Universal Edital 15/2007, 476808/2007-3), FIOCRUZ/PAPES V
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Received 31 January 2012
Accepted 29 June 2012

tis A viral titre was estimated by a quantified real-time polymerase chain reaction (RT-PCR) technique for detecting negative-strand intermediate RNA as described previously by de Paula et al. (2007). The RT-PCR was carried out using the forward primer (5'-CTGCAGGTTCAGGGTTCTTAAATC-3'), the reverse primer (5'-GAGAGCCCTGGAAGAAAGAAGA-3') and the HAV-probe (FAM 5'-ACTCATTTTTCACGCTTTCTG-3'). All PCRs were performed using the 7500 Real-Time PCR System (Applied Biosystems). For each PCR run, a master mix was prepared at 0°C with 12.5 µL of 1x TaqMan Universal PCR Master Mix (Roche, Nutley, NJ, USA), 1.25 µL of assay by design (300 nM of each primer and 150 nM of probe, Gentec assay, Applied Biosystems), 4 µL of DNAse/RNAse free water and 5 µL of cDNA. The thermal cycling conditions consisted of denaturation for 10 min at 94ºC followed by 40 cycles of 15 sec at 94ºC and a final 1 min cycle at 60ºC.

The preparation and characterisation of chicken anti-HAV IgY was performed in a previous study (de Paula et al. 2010) according to a method established in our laboratory. The experimental protocol was reviewed and approved by the Ethical Commission for the Use of Animals (University Centre Serra dos Órgãos, protocol 0272/2009). In brief, clinically healthy Isa brown laying chickens were immunized intramuscularly with 100 µL of HAV (10^5 copies/mL), associated with 20 µL of CpG oligodeoxynucleotide adjuvant (1 mg/mL) (5'-TCGTCGTTTGTCGTTTTGTT-3', Integrated DNA Technologies, USA) and 100 µL of incomplete Freund's adjuvant (Sigma-Aldrich, Saint Louis, MO, USA). Chicken eggs were collected daily and the egg yolks were purified using polyethylene glycol precipitation as described in Polson et al. (1985). The presence of IgY was determined by spectrophotometry using the Lowry method. Additionally, the presence of anti-HAV IgY was assessed by western blotting and the specificity and titre of anti-HAV IgY were confirmed by a commercial immunoenzymatic assay Bioelisa HAV (Biokit, Barcelona, Spain). After characterisation, anti-HAV IgY was conjugated to horseradish peroxidase (HRP) using the method described by Nakane & Kawaoi (1974) and modified by Camargo et al. (1987).

Anti-HAV IgY was assessed using a direct *"*in-house*"* immunoenzymatic assay for the detection of HAV. Initially, a plate (C96 Microwell plate, Nunc, New York, USA) was coated with 100 µL of capture anti-HAV IgY (1:1000) diluted in phosphate buffered saline (PBS) and incubated overnight at 4ºC. The plate was then washed five times with 220 µL of PBS containing 0.5% Tween-20 (PBS-T). A total of 200 µL blocking buffer (PBS-T + BSA 5%) was then added to the plate, which was incubated at room temperature for 1 h. After the incubation, the wells were washed with 220 µL of PBS-T and 50 µL of either PBS or HAV (10^5 copies/mL) was added to the wells. The plate was incubated for 1 h at 37ºC and washed as described above. The HRP-conjugated antibody was then added in duplicate at dilutions of 1:100, 1:250, 1:1000 and 1:2000 and the plate was incubated for 1 h at 37ºC. Following this incubation, the plate was washed, 100 µL of tetramethylbenzidine dihydrochloride (TMB) (Sigma-Aldrich, USA) was added and the plate was incubated for 10 min. The reaction was terminated by adding 100 µL of sulphuric acid (2.0 M) and the absorbance of the wells was read at an optical density of 450 nm (OD_{450}).

To evaluate the optimal concentration of anti-HAV IgY as capture and conjugated antibodies, we performed a competitive immunoenzymatic assay in which the HRP-conjugated anti-HAV IgY competed with the total anti-HAV immunoglobulin content of the samples. The plates (Maxisorp, USA) were coated with 100 µL capture anti-HAV IgY antibody diluted 1:1000 and incubated overnight at 4ºC. The wells were then washed five times with 220 µL PBS-T. After the wash, 200 µL PBS was added to half of the wells and 200 µL blocking buffer was added to the other half. This step was performed to determine whether the anti-HAV IgY immunoenzymatic assay is better optimized with or without the blocking step. The plates were incubated at room temperature for 1 h, followed by another wash. Then, 50 µL of HAV (10^5 copies/mL) and 50 µL of positive control (serum containing IgG anti-HAV) or negative control (serum without IgG anti-HAV) diluted 1:10 or 1:100 were added to the wells. The plates were incubated at 37ºC for 1 h, followed by another wash. After the wash, 100 µL of the HRP-conjugated anti-HAV IgY was added at dilutions of 1:250, 1:500 and 1:1000. The plates were incubated at 37ºC for 1 h and then washed. After the wash, 100 µL of TMB was added and the plate was incubated for 10 min. The reaction was stopped with 100 µL of sulphuric acid (2.0 M) and the well absorbance was read at OD_{450}.

A serum panel consisting of 100 positive and 81 negative serum samples, confirmed by an anti-HAV IgG competitive immunoenzymatic assay (Biokit, Barcelona, Spain), collected in the year of 2009 from outbreak volunteers (from the city of Três Rios, Rio de Janeiro, Brazil), was utilized for evaluation of the anti-HAV IgY competitive immunoenzymatic assay. The utilization of volunteers' was approved by the Ethical Committee of Oswaldo Cruz Foundation (CEP/FIOCRUZ) (reference 536/09). Each sample from the serum panel was tested in duplicate in an *"*in-house*"* anti-HAV IgY immunoen-

TABLE I

Optical density of 450 nm (OD_{450}) values of various dilutions of horseradish peroxidase (HRP)-conjugated immunoglobulin Y used in a direct immunoenzymatic assay

HRP-conjugated dilution	OD_{450}	
	Negative[a]	Positive[b]
1:100	1.376	3.901
1:250	0.678	3.392
1:1.000	0.267	1.161
1:2.000	0.234	0.687

a: wells with phosphate buffered saline; *b*: wells with hepatitis A virus.

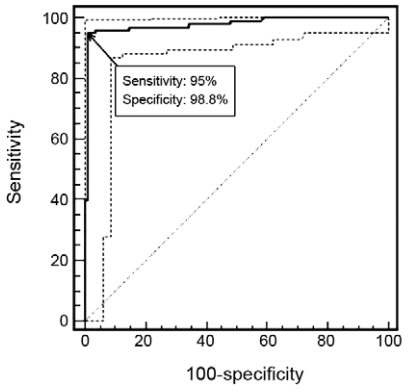
zymatic assay, as described previously. The statistical analysis for the assay was performed with the MedCalc program (version 9.3.9.0).

The results showed an average IgY concentration of 19.3 mg/mL in the chicken yolk, which was greater than the average value found in the rabbit serum (7.5 mg/mL). The commercial immunoenzymatic assay revealed that all of the chicken eggs had anti-HAV IgY immunoglobulin with a maximum titre of 1:1000 and western blotting confirmed the presence of anti-HAV IgY in the egg yolks.

Anti-HAV IgY efficiency was determined through the greatest distinction between the OD_{450} of the positive sample (virus) and that of the negative control (PBS). The optimal dilution of the HRP-conjugated anti-HAV IgY antibodies was 1:1000 (Table I). The average OD_{450} of the positive control was 1.1615 and 0.292 for the negative control, indicating a low background. Our results demonstrated that IgY can bind to HAV in standardisation assays and that anti-HAV IgY can be used for HAV detection in a direct immunoenzymatic assay using HRP-conjugated IgY at a 1:1000 dilution and IgY as a capture antibody at a 1:1000 dilution; these same dilutions were used in *"*in-house*"* assays that utilize human antibodies for the detection of total human anti-HAV antibodies (Vitral et al. 1991).

For standardization of the competitive anti-HAV assay, the optimal dilution of the samples and HRP-conjugated antibodies and the optimal difference between the OD_{450} of the positive and negative samples for anti-HAV IgY were determined. Sample dilutions of 1:100 and HRP-conjugated antibody dilutions of 1:250, 1:500 and 1:1000 were used in this assay. The sample dilution of 1:10 was only used with the HRP-conjugated antibody dilution of 1:250. This was realized according to the dilution utilized in the commercial assays for identification of total anti HAV and according to a previous assay (data not show) wich demonstrated a good OD_{450} difference between the positive and negative samples with a HRP-conjugated antibody dilution of 1:250. The greatest OD_{450} difference between the positive and negative samples occurred at a serum sample dilution of 1:100 and a 1:1000 dilution of the capture antibodies and HRP-conjugated anti-HAV IgY (Table II). These dilutions were utilised in the anti-HAV IgY competitive immunoenzymatic assay. The efficacy of HRP-conjugated anti-HAV IgY in our assay was similar to that of IgG in a similar *"*in-house*"* assay (Vitral et al. 1991). This efficacy occurs because the optimal dilution of HRP-conjugated anti-HAV IgY is 1:1000, which is the same as the dilution of IgG HRP-conjugated antibodies used normally in an *"*in-house*"* assay to detect the total anti-HAV immunoglobulin content. Additionally, the volume of serum necessary for the assay using IgY was 10-fold less than the serum needed for the commercial assay using IgG.

An average OD_{450} cut-off of 0.6611 was encountered in the serum panel, this value was similar with the average cut-off encountered in the commercial immunodiagnostic assay (OD_{450} of 0.655). The results from the statistical analysis of serum panel were obtained according to the optimal precision (to minimize the occurrence of false negatives and false positives) obtained by analysis of the receiver operator curve (Figure). Statistical analysis of the assay demonstrated a sensitivity of 95% and a specificity of 98.8%. In general, for an immunoenzymatic assay to be considered a satisfactory assay, the



Receiver operator curve demonstrating sensitivity and specificity of the "in-house" immunoenzymatic assay using anti-hepatitis A virus immunoglobulin Y.

TABLE II

Optical density of 450 nm values of various dilutions of horseradish peroxidase (HRP)-conjugated immunoglobulin Y and serum with or without the blocking step in an "in-house" competitive immunoenzymatic assay

HRP-conjugated dilutions	Serum dilutions	Serum without blocking step		Serum with blocking step	
		Positive[a]	Negative[b]	Positive[a]	Negative[b]
1:250	1:10	1.148	1.414	0.801	1.328
	1:100	1.281	2.338	0.887	2.099
1:500	1:100	0.756	1.383	0.509	1.205
1:1.000	1:100	0.478	0.911	0.340	0.841

a: wells with positive serum samples using immunoglobulin G (IgG) anti-hepatitis A virus (HAV); *b*: wells with negative serum samples using IgG anti-HAV.

sensitivity and specificity must be above 90% (Camargo et al. 1987). The likelihood ratio indicates how variable the result of a diagnostic assay is capable to change the probability that a person has the disease in question. Our results demonstrated a positive "likelihood ratio" (LR+) of 76.95 and a negative "likelihood ratio" (LR-) of 0.05, both results demonstrated that the assay has a high probability to confirm (LR+) or exclude (LR-) the presence of disease. The positive predictive value was 99% and the negative predictive value was 94.1%. The predictive values are defined as the precision of the assay and predictive values above 80% are considered very reasonable values for an immunoenzymatic assay (Camargo et al. 1987).

In conclusion, the statistical analysis of the anti-HAV IgY competitive immunoenzymatic assay demonstrated that anti-HAV IgY antibodies can be utilised in a competitive immunoenzymatic assay for the detection of anti-HAV antibodies. This assay appears to be a sufficiently sensitive and feasible assay for the detection of total anti-HAV antibodies in human serum. This assay could be a good alternative for the diagnosis of hepatitis A because it has the similar specificity as commercial immunoenzymatic assays and the antibody collection method occurs by purifying egg yolks, which reduces animal suffering. To our knowledge, this is the first study that utilizes IgY in an immunoenzymatic assay against a virus that infects humans.

REFERENCES

Camargo IF, Gaspar AMC, Yoshida CFT 1987. Comparative ELISA reagents for detection of hepatitis B surface antigen (HBsAg). *Mem Inst Oswaldo Cruz 82*: 181-187.

Carlander D, Kollberg H, Larsson A 2002. Retention of specific yolk IgY in the human oral cavity. *BioDrugs 16*: 433-437.

Costas L, Vilella A, Trilla A, Serrano B, Vera I, Roldan M, Sancho MP, Bayas JM, Gascon J, Costa J 2009. Vaccination strategies against hepatitis A in travelers older than 40 years: an economic evaluation. *J Travel Med 16*: 344-348.

Cova LJCV 2005. DNA-designed avian IgY antibodies: novel tools for research, diagnostics and therapy *34* (Suppl.): S70-S74.

de Paula VS, da Silva AD, de Vasconcelos GA, Iff ET, Monteiro Silva ME, Kappel LA, Cruz PB, Pinto MA 2010. Applied biotechnology for production of immunoglobulin Y specific to hepatitis A virus. *J Virol Methods 171*: 102-106.

de Paula VS, Diniz-Mendes L, Villar LM, Luz SL, Silva LA, Jesus MS, da Silva NM, Gaspar AM 2007. Hepatitis A virus in environmental water samples from the Amazon Basin. *Water Res 41*: 1169-1176.

Kummer A, Li-Chan EC 1998. Application of an ELISA-elution assay as a screening tool for dissociation of yolk antibody-antigen complexes. *J Immunol Methods 211*: 125-137.

Kvietkauskaite R, Acaite J 2004. Evaluation of an ELISA for detection of antibodies to sendai virus in swine sera. *Turk J Vet Anim Sci 28*: 373-376.

Lei JH, Liu WQ, Sun CS, Tang CL, Li MJ, Chen YL, Li YL 2009. Detection of circulating antigen in serum of mice infected with *Schistosoma japonicum* by immunomagnetic bead ELISA based on IgY. *Acta Trop 111*: 39-43.

Leung AK, Kellner JD, Davies HD 2005. Hepatitis A: a preventable threat. *Adv Ther 22*: 578-586.

Nakane PK, Kawaoi A 1974. Peroxidase-labeled antibody. A new method of conjugation. *J Histochem Cytochem 22*: 1084-1091.

Polson A, Coetzer T, Kruger J, von Maltzahn E, van der Merwe KJII 1985. Improvements in the isolation of IgY from the yolks of eggs laid by immunized hens. *Immunol Invest 14*: 323-327.

Saha SK, Saha S, Shakur S, Hanif M, Habib MA, Datta SK, Bock HL 2009. Community-based cross-sectional seroprevalence study of hepatitis A in Bangladesh. *World J Gastroenterol 15*: 4932-4937.

Schade R, Calzado EG, Sarmiento R, Chacana PA, Porankiewicz-Asplund J, Terzolo HR 2005. Chicken egg yolk antibodies (IgY-technology): a review of progress in production and use in research and human and veterinary medicine. *Altern Lab Anim 33*: 129-154.

Sunwoo HH, Wang WW, Sim JS 2006. Detection of *Escherichia coli* O157:H7 using chicken immunoglobulin Y. *Immunol Lett 106*: 191-193.

Veerasami M, Singanallur NB, Thirumeni N, Rana SK, Shanmugham R, Ponsekaran S, Muthukrishnan M, Villuppanoor SA 2008. Serotyping of foot-and-mouth disease virus by antigen capture-ELISA using monoclonal antibodies and chicken IgY. *New Microbiol 31*: 549-554.

Victor JC, Monto AS, Surdina TY, Suleimenova SZ, Vaughan G, Nainan OV, Favorov MO, Margolis HS, Bell BP 2007. Hepatitis A vaccine versus immune globulin for postexposure prophylaxis. *N Engl J Med 357*: 1685-1694.

Villar LM, da Costa MCE, de Paula VS, Gaspar AMC 2002. Hepatitis a outbreak in a public school in Rio de Janeiro, Brazil. *Mem Inst Oswaldo Cruz 97*: 301-305.

Vitral CL, Gaspar AM, Yoshida CF 1991. Two competitive enzyme immunoassays for the detection of IgG class antibodies to hepatitis A antigen. *Rev Soc Bras Med Trop 24*: 79-85.

Wheeler C, Vogt TM, Armstrong GL, Vaughan G, Weltman A, Nainan OV, Dato V, Xia G, Waller K, Amon J, Lee TM, Highbaugh-Battle A, Hembree C, Evenson S, Ruta MA, Williams IT, Fiore AE, Bell BP 2005. An outbreak of hepatitis A associated with green onions. *N Engl J Med 353*: 890-897.