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• VIRAL HEPATITIS •

Detection of anti-HAV antibody with dot immunogold filtration assay

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Abstract

AIM: To establish a rapid, sensitive and specific immunogold assay for detection of hepatitis A virus infection.

METHODS: Rabbit monoclonal antibodies to anti-human IgM and IgG (Dako) were dotted on a nitrocellulose membrane (NCM) respectively to capture the human sera IgM and IgG. Then the captured antibodies would conjugate to HAV antigen, which was revealed by mouse anti-HAV IgG conjugated to gold particles. Final results were assessed by blind method.

RESULTS: Sera from 96 patients with acute hepatitis were used for our study. Compared with well-recognized standard (Abbott Laboratory, USA), the sensitivity and specificity of IgM-DIGFA (self-made) were 91.3 % (42/46) and 96.0 % (48/50), and those of IgM-ELISA (Kehua, Shanghai) were 97.8 % (45/46) and 100.0 % (50/50). The identical results were produced from the study with reagents at different conditions, and the study was repeated in 15 negative sera and 10 positive sera. The serum anti-HAV IgG was tested with DIGFA at the same time. In comparison with ELISA, the sensitivity and specificity of DIGFA for IgG anti-HAV were 87.2 % (41/47) and 91.8 % (45/49), respectively.

CONCLUSION: This assay can detect anti-HAV IgM and IgG simultaneously, and be done within 3 minutes. The simplicity, rapidity and specificity of the assay were useful for screening and epidemiological study.

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INTRODUCTION

Hepatitis A is a self-limiting disease and often a subclinical disorder[1,2]. Since symptomatic hepatitis A infection can be clinically undistinguished from hepatitis B, C or E, serological testing is an important tool in its diagnosis[3-6]. Diagnosis of HAV infection depends mainly on the detection of specific antibody[3,4]. Although enzyme linked immunosorbent assay (ELISA) and RT-PCR are currently used for the detection of HAV infection and meet most of the clinical requirements[7-11], the two methods provide little information on prevention of diseases[12-15]. Dot immunogold filtration assay for the detection of anti-HAV IgM was established[16,17]. In our study a dot immunogold filtration assay was established to detect both anti-HAV IgM and IgG simultaneously.

MATERIALS AND METHODS

Sera

Blood samples were continuously collected from acute hepatitis inpatients in Xi' an Infectious Disease Hospital from March to October 2000 with permission of both the hospital and patients. Five milliliters of blood were drawn from each patient and the specimen was centrifuged at 3 000 r/min for 15 minutes to separate the serum. Ninety-six sera specimens were collected by the end of study. Anti-HAV IgM was detected as positive in 46, anti-HBc IgM in 31, anti-hepatitis C in 15, transmission-transmitted virus (TTV) in 3 by RT-PCR[18]. No hepatitis virus markers were found in 6 specimens, and positive anti-HAV and HBV were detected simultaneously in 2 sera.

Preparation of probe

Colloidal gold was produced by citromalic acid trisodium recovery method. The colloidal gold solution was scanned between 400 nm and 700 nm by using a spectrophotometer. The batch with λmax between 519 and 520 was used subsequently for conjugating with mouse anti-HAV IgG (CAPM). The pH was adjusted to 8.0 with 0.2 mol/L K_2CO_3. Then mouse anti-HAV IgG (1 μg per milliliter) was added into the colloidal gold solution and mixed for 30 minutes, then stored at 4 ℃ overnight. The pellet was collected by centrifugation at 15 000 r/min for 60 minutes, and the absorbance (A) was regulated by 0.02 mol/L PBS to the value of 1.5.

ELISA test for anti-HAV IgM and IgG

ELISA test was performed strictly according to instructions of the kit (anti-IgM kit from Kehua Biotech Co. and anti-IgG kit from Huaguang Biotech Co). Diluted sera (1:1 000) were incubated at 37 ℃ for one hour in a reactive well, and after washed, HAV Ag and anti-HAV peroxidase conjugates were added and incubated at 37 ℃ for 10 minutes, and followed by washing. Then the substrate was added, and the reaction was stopped by diluted sulfuric acid 10 minutes later. The absorbance was measured at 540 nm. An absorbance of 2.1 times the negative control value was considered as positive. The detecting procedures for HAV IgG were the same as for IgM except for serum dilution (1:50).

AXSYM HAVAB-M (Abbott Laboratory) was based on microparticle enzyme immunoassay technology[19-21]. Samples and all AXSYM HAVAb-M reagents required for one test were pipetted with the sampling probe into wells in reactive vessel in the sampling center. The reaction vessel was immediately

transferred into the processing center. Further pipetting was done in the processing center by the processing probe. All steps were completed automatically and the diagnostic results were reported immediately.

DIGFA

There were 4 components in the kit: solid phase reaction board (SPRD, self-made), HAV Ag, color-developing reagents and lotion. Anti-human IgM and IgG antibodies (Dako, USA) were blotted onto small round nitrocellulose membranes (Hyclone, USA) separately, air-dried at room temperature, and then incubated in 20 g/L bovine serum albumin (BSA) overnight, and finally washed and air dried. These nitrocellulose membranes prepared were fitted in SPRD with coated side facing exteriorly. Then SPRD was filled with water-absorbed stuff. One drop of lotion (PBS-T, pH8.0 containing 05 g/L Tween20 and 20 g/L BSA) was added to prepare SPRD. 100 μL of serum with dilution of 1:100 was added to each reaction well, and then washed with one drop of lotion. Then one drop of HAV Ag was added, and then was washed. Finally two drops of anti-HAV IgG colloidal gold conjugates were added, and then washed with one drop of lotion. A reddish dot with sharp margin was considered as positive.

Statistical analysis

The following definitions and formulae were used in this study. A true positive (a) sample was both reactive by DIGFA/ELISA and ABBOTT. A true negative (d) sample was nonreactive by both DIGFA/ELISA and ABBOTT. A false positive (b) sample was reactive by DIGFA/ELISA but negative by ABBOTT and a false negative (c) sample was negative by DIGFA/ELISA but positive by ABBOTT. The sensitivity of DIGFA was defined as the probability that a sample containing anti-HAV antibodies would be positive in DIGFA/ELISA. The specificity of DIGFA/ELISA was defined as the probability that a sample without anti-HAV antibodies would be negative in DIGFA/ELISA. Chi-square test was used.

RESULTS

Preparation of probe

Transmission electron microscopic studies revealed that the average diameter of the gold particle was 15 nm (15±0.7 nm). The maximum absorbing wavelength was 519 nm, indicating that the colloid gold met the experimental requirements. After purification of labeling anti-HAV IgG to gold particle, one drop of probe was added to SPRD coated by goat anti-mouse IgG and a pink dot appeared 30 seconds later, indicating effectiveness of the probe.

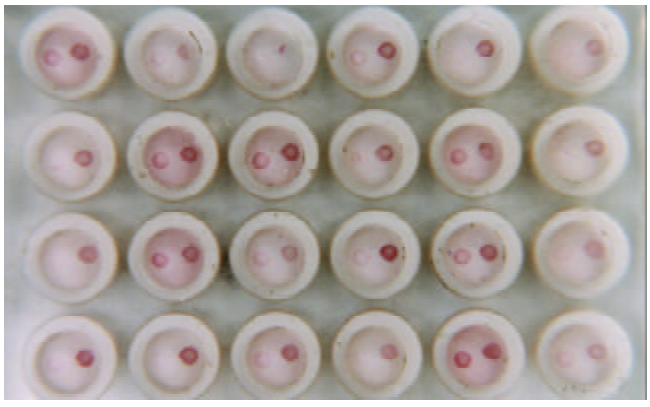

Figure 1 Detection of 24 sera from acute hepatitis inpatients by DIGFA. Red dot: positive; Left: anti-human IgG; Right: anti-human IgM.

Comparison of three diagnostic methods (Tables 1, 2 and 3)

Abbott kit has been widely recognized in the world, but its usage was limited in China due to the relatively high cost. The comparative analysis of 96 serum specimens showed that the sensitivity and specificity of IgM-ELISA were higher than those of IgM-DIGFA (Table 1). Statistical analysis (two tailed binomial probability test) showed no significant differences in sensitivity and specificity (Figure 1). Cross-reaction was found in DIGFA for anti-HAV IgM in a serum specimen, which was diagnosed as anti-HBV positive by ELISA.

Table 1 Comparison of DIGFA, ELISA and AXSYM HAVAb-M

IgM		HAVAB-M (Abbott laboratory)		Total
		+	-	
DIGFA	+	42	2	44
	-	4	48	52
ELISA	+	45	0	45
	-	1	50	51

Sensitivity of IgM-DIGFA was 91.3 % (42/46) and specificity was 96.0 % (48/50). Sensitivity of IgM-ELISA was 97.8 % (45/46) and specificity was 100.0 % (50/50). There were no significant differences in sensitivity (P=0.36) and specificity (P=0.49).

Anti-HAV IgG and IgM were detected in all sera. ELISA kit was purchased from Huaguang Biotech Co, Xi' an, China. Forty-one of 96 sera specimens were detected as positive with both methods and 86 were diagnosed coincidentally. The sensitivity and specificity were 87.2 % (41/47) and 91.8 % (45/49), respectively (Table 2).

Table 2 Comparison of IgG-DIGFA with IgG-ELISA

IgG-DIGFA	IgG-ELISA		Total
	+	-	
+	41	4	45
-	6	45	51
Total	47	49	96

In this study, twenty-three sera specimens were detected as positive for IgG, and 30 as negative for both IgM and IgG, which indicated that some individuals in general population were possibly susceptible to HAV infection (Table 3).

Table 3 Analysis of anti-HAV IgG and IgM by DIGFA method

Anti-HAV IgG	Anti-HAV IgM		Total
	+	-	
+	23	22	45
-	21	30	51
Total	44	52	96

Repeatability and stability

All reagents could be stored effectively at 4 ℃ for 3 months, at room temperature for 15 days and at 48 ℃ for 2 days. Fifteen negative and ten positive sera samples were selected randomly and detected with reliable results.

DISCUSSION

Detection of antibody against hepatitis A virus

DIGFA is a new technique of solid phase labeled immunoassay,

in which NCM is used as a support and colloidal gold as the label, and the principle of filtration is adapted for the rapid reaction of antigen and antibody[22,23]. The use of visible gold-conjugated monoclonal antibody instead of enzyme in ELISA makes the test simple[24]. The sensitivity of DIGFA is lower than that of ELISA. This method can detect two kinds of anti-HAV antibodies simultaneously that are similar to protein biochip, but the cost is much lower than that of biochip[25]. It is very suitable for screening and vaccination program[26-29]. IgM antibody persists for 3 to 6 months afterwards, and is seldom found after vaccination. Patients with asymptomatic hepatitis A may have detectable anti-HAV IgM for a shorter period than patients with symptomatic diseases[30]. Although anti-HAV IgG may be present at early stage of infection, it is always accompanied by anti-HAV IgM at the onset of illness. Anti-HAV IgG alone indicates a past infection and persists for decades after acute HAV infection, reflecting recovery and resistance to reinfection[31,32]. However, detection of anti-HAV IgG is often ignored due to its limited clinical value, but it can show whether a person is susceptible to HAV and offer a good instruction to health workers. Twenty-two sera were detected as negative for both anti-HAV IgM and IgG, which indicated that these people need to be vaccinated.

Comparison with ELISA

The sensitivity and specificity of IgM-DIGFA for HAV in these sera were lower than those of IgM-ELISA. However, there was no significant difference in specificity of both assays. IgM-DIGFA can be used as an eligible assay to screen HAV infection for its rapidity and specificity. It takes a few minutes to complete the whole process by DIGFA, and the detection of 30 sera specimens can be done within 30 minutes. Shortened reaction time gives a proper explanation to the lower sensitivity of IgM-DIGFA. A cost-analysis of DIGFA and ELISA was done, and found that pure reagents cost of DIGFA was 0.75 Yuan (RMB) and that of anti-HAV IgM ELISA was 1.20 Yuan (RMB). It was not as expensive as expected and two kinds of antibodies were detected at one time[33]. All the reagents leaked through NCM and took a full reaction with antibodies attached to NCM. This was due to the effect of immunoconcentration [34]. It is easy to tell the results without the help of extra apparatus. Therefore, it can be widely used in an epidemiological survey.

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