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Detection of Antibody to Hepatitis Delta Virus in Human Serum by Double Antigen Sandwich ELISA

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Abstract: A simple rapid detection of antibody to hepatitis delta virus (anti-HDV) in human serum was developed by using double antigen sandwich ELISA. HDV gene fragment encoding HDAg was isolated from a Chinese patient infected with HDV by RT-PCR, and a high-efficient expression HD-PQE31 strain was constructed with the fragment. We obtained high titer and good quality hepatitis delta virus protein purified by Ni-NTA metal-affinity chromatography, which was identified by Western blot and ELISA, then we set up the double antigen sandwich ELISA for detection of anti-HDV in human serum, and the performance of the sandwich ELISA was evaluated in terms of specificity and sensitivity. Results were: 1) The purified HDAg protein's purity was 90%, and its ELISA titer was 1/100 000. 2) 42 anti-HDV positive sera were detected and showed that the sensitivity of sandwich ELISA was higher than that of competitive ELISA (t=2.44, p<0.01). 3) The inhibitory rates for 2 anti-HDV positive sera by the specific HDAg were 74% and 93% respectively. 4) For the assay of specificity, all 60 samples infected by other hepatitis viruses and 30 normal samples were negative for anti-HDV. These results suggested that the double antigen sandwich ELISA with purified recombinant HDAg showed higher specificity and sensitivity, It can be used in routine laboratories to diagnose the HDV infection.

key words: Hepatitis delta virus; Hepatitis delta virus antigen; Anti-HDV; Sandwich; ELISA

Hepatitis delta virus (HDV) is a single-stranded, ringed negative-sense small RNA virus with a genome of approximately 1700 bases (6). It replicates only in patients who are concurrently infected with hepatitis B virus (HBV) (2). Concurrent infection by HBV and HDV increases the risk of severe liver disease compared to infection with HBV alone; the liver cells can be seriously damaged and the incidence of chronic hepatitis, hepatocirrhosis and hepatocellular carcinoma is higher than for other types of hepatitis (16, 17). There is a high incidence of HDV within the country an it is very important to establish sensitive and specific assays for HDV diagnosis in a timely manner. Antibody to hepatitis delta virus (anti-HDV) is one of

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the primary diagnostic HDV markers. This assay is the usual method for diagnosis of HDV infection since viremia lasts only a few weeks, and will help to determine the stage of the disease, degree of infectivity, prognosis, and immune status of the patient.

Up to now, anti-HDV has been measured by competitive ELISA, in which HDAg and detected sample are added to an antibody-coated well (3); there are also some other methods but they generally require kits which are manufactured by foreign companies and are often too expensive for routine use in developing countries (1,12). In addition, the HDAg for preparation of anti-HDV kit mainly came from animals infected by HDV and this can be restrictive for quality control (2). In the study, we have established a double antigen sandwich Enzyme-linked Immunosorbent Assay (ELISA) protocol for detection of anti-HDV in human serum by producing high-efficient purified and high titer recombinant HDAg protein, and we have evaluated the new assay by comparison with competitive ELISA and indirect ELISA.

MATERIALS AND METHODS

Samples and sera

30 normal samples from healthy subjects, negative for HAV, HBV, HCV, HDV and HEV, were used as negative controls.

42 anti-HDV-positive samples were collected from anti-HDV-positive patients confirmed by Beijing Hepatitis Institute's anti-HDV kit (competitive assay). Of these samples, 12 were chronic HDV infections, 26 were cirrhosic and 4 had hepatocellular carcinomas; there were 29 males and 13 females, median age was 48 years (range, 27 to 77 years).

Of the 60 samples infected by other hepatitis viruses, there were 50 HAV IgM, 2 anti-HBs, 2 anti-HBe, 2 anti-HBc, 2 anti-HCV and 2 anti-HEV positive samples, all of them were HDAg, anti-HDV and immunoglobulin M anti-HDV antibody (IgM anti-HDV) negative.

All sera were collected from patients at Beijing You-an Hospital, affiliated with the Capital University of Medical Sciences, between January 1997 to October 2005, and stored at -80°C until tested; they were diagnosed according to the standards of the 10th National Infection and Verminosis Symposium at Xi'an in 2000.

Reagents

The HBV markers kit (comprising: HBsAg, anti-HBs, HBeAg, anti-HBe, and anti-HBc) and the anti-HCV kit were purchased from Abbott, the anti-HDV kit including competitive ELISA and indirect ELISA (3), HDAg kit and anti-HEV kit were developed by the Beijing Hepatitis Institute. Anti-HDV positive serum used as positive control and HDAg used as reference were from Professor I. D. Gust(Fairfield Hospital, Melbourne, Australia); recombinant HDAg (pD280) was donated by the Research Laboratory of Hepatitis, Institute for Viral Disease Control and Prevention, Chinese Center for Disease Control and Prevention. It was purified by Sephadex G200 and its ELISA titer was 1/1 000. At the initiation of the study, negative controls were from the anti-HDV kit purchased from Noctech Ltd., Dublin, reland since then we have collected the sera from healthy subjects in our country and tested them for anti-HDV with anti-HDV kit(Noctech Ltd., Dublin, Ireland), using the anti-HDV negative sera as negative controls. HRP was purchased from Sigma; HRP conjugation of goat anti-mice IgG was from Dingguo Biotechnology

Company (Beijing, China); it was used in an indirect assay for testing anti-HDV. M-MLV Reverse Transcriptase was from GibcoBRL (USA), RNase Inhibitor, Tag DNA polymerase and Random Primers were purchased from Shanghai Sangon Biological Engineering & Technology and Service Co. Ltd. (China), T_4 DNA Ligase and restriction endonucleases (*Hin*d Ⅲ, *Bam*HⅠ) came from TaKaRa Biotechnology (Dalian, China).

Confirmation of positive sera

The mean negative control absorbance value was calculated in the following manner. The mean negative control optical density (*OD*) value must be less than or equal to 0.10, if more than one negative control *OD* value does not meet the criteria, the run is invalid and must be repeated. The mean positive control *OD* value was calculated as follows. The mean positive control OD value must be greater than or equal to 0.21, if not, the run is invalid and must be repeated. The presence or absence of HDAg is determined by comparing the *OD* value of the unknown samples to that of the *OD* value of the negative control, samples were confirmed to be positive with P/N≥2.1(P: *OD* value of sample, N: *OD* value of negative control) (15).

Preparation and purification for HDAg

The HDV gene fragment with a length of about 340 base pair (bp) was amplified from a HDV patient's serum by reverse transcription and nested polymerase (RT-PCR), the outer primer (primer HD1: 5'-AGAGGAAAGAAGGACGCGAGAC-3', 906→927, positive pole; primer HD4: 5'-AAGAGTAAGAGC ACTGAG GA-3', 1640→1620, negative pole) and inner primer (primer HD2: 5'-TCTTGTTCTCGAGG GCCTT-3', 1268→1286, positive pole; primer HD3: 5'-ATATGGATCCGTCGAAGATGAGCCGGTCC-3', 1608→1590, negative pole) were 735 bp and 341 bp respectively, with primer HD3 introducing the cleavage site (GGATCC) for *Bam*HⅠ.

The PCR products were reclaimed with low melting point gel and inserted into expression vector PBluescript Ⅱ KS. After double digestion cleaving by *Hin*d Ⅲ and *Ba*mHⅠ, the HDV gene fragment was linked to PQE_{31} vector and then transformed into *E. coli* M15 to construct expression vector HD-PQE_{31}.

The expression vector HD-PQE_{31} was proliferated, inducted with isopropyl-β-D-thiogalactoside (IPTG), lysed by lysozyme, and disrupted by ultrasonic. Then the recombinant protein was purified by nickel-nitrilotriacetic acid (Ni-NTA) metal-affinity chromatography (Qiagen, Netherlands), and was identified for its purity and ELISA titer by SDS-PAGE assay and ultraviolet scan(AlphaImager HP, Alphainnotech Corp., CA,USA) and ELISA with the professor Gust's HDAg as control respectively.

Preparation of HRP-conjugated HDAg

The purified HDAg protein was conjugated to HRP using the standard periodate sodium method.

ELISA for anti-HDV

Microtiter plates (Nunk, Roskilde, Denmark) were coated with 0.25μg, 0.5μg and 1μg HDAg in 100μL carbonate coating buffer per well and allowed to sit overnight at 4℃. The plates were washed three times with 0.02 mol/L phosphate-buffered saline (PBS, pH7.2) containing 0.05% Tween 20. Then the plates were blocked with blocking solution (1% bovine serum albumin in PBS, pH7.2) at 37℃ for 2h, and washed three times after removal of the blocking solution. 5μL specimen was mixed with 95μL lysis solution (pH7.2, PBS containing 2% *E. coli* to dissociate the envelope of HDV), and 100μl of the pretreatment

solution was added to each well. The plates were incubated for 90 min at 37℃ and then washed three times with the same washing buffer. HRP-conjugated HDAg was diluted at 1/4 000 with dilution buffer containing 10% goat serum and added to each well, the plates were incubated for 60 min at 37℃. After the plates were washed three times with the same washing buffer, 100μL of tetramethylbenzidine (TMB) substrate solution was added and incubated for 15 min at 37℃; the reaction was then stopped with 100μl of 1 mol/L sulfuric acid. Then the *OD* values were measured at 450nm, with 630nm as the reference wavelength, by using a microplate reader (BioRad 550, Bio-Rad, USA).

Selection of the coated HDAg. First, the purified HDAg was coated at concentrations of 2.5μg/mL, 5μg/mL and 10μg/mL. Second, an equimolar mixture of the purified HDAg and the HDAg (pD280) were coated at a total concentration of 10μg/mL, and then the test was carried out as described above. In this assay, 4 anti- HDV positive sera were serially diluted at 1/10, 1/40, 1/80, 1/160, and, along the one anti-HDV negative serum, were compared with the two kinds of coated plates.

Validation of the double antigen sandwich ELISA

All the 42 anti- HDV positive sera were serially diluted at 1/2, 1/20, 1/40, 1/80, 1/160, 1/320, then assayed with the double antigen sandwich ELISA and the anti-HDV kit (competitive ELISA) developed by Beijing Hepatitis Institute.

Neutralization assay. Each of the 2 anti- HDV positive sera was diluted at 1/100, 1/300, 1/900, and neutralized by the addition of equal volume of the purified HDAg, then tested with the double antigen sandwich ELISA.

42 anti-HDV positive sera were tested for HDV RNA with RT-PCR according to the protocol described elsewhere (4).

To evaluate the specificity of the assay, 2 positive controls of anti-HDV positive serum, 60 other type hepatitis serum and 30 normal sera were tested with the sandwich ELISA.

Statistical Analysis

Correlation between the double antigen sandwich ELISA and the competitive ELISA was analysed using a t-test to determine statistical significance.

RESULTS

Preparation and purification for HDAg

We amplified the HDV gene fragment with 340 bp by RT-PCR for efficient expression of HDAg which primarily existed in inclusion bodies. In SDS-PAGE assay, the result was a single band and showed the molecular weight of the purified HDAg to be ~16kDa; the specific identification of purified HDAg protein showed a clear positive reaction with anti-HDV HRP, and the purified HDAg protein's purity was 90% by ultraviolet scan. After reversion from denaturalization, the concentration of the purified HDAg protein was 0.5mg/mL, and its ELISA titer was 1/100 000.

ELISA for anti-HDV

In the selection of the coated HDAg, the results showed that both 5μg/ml and 10μg/mL gave clear signals, so we selected a concentration of 5μg/mL.

For the testing of four anti- HDV positive serial sera and one anti- HDV negative serum, *OD* values at plates coated with purified HDAg were higher than those observed with plates coated with a mixture of purified HDAg and HDAg (pD280) (data not shown), so we selected the first coating model.

Table 1. Positive numbers for 42 anti- HDV positive serial sera with two assays

Dilution ratio	1/2	1/20	1/40	1/80	1/160	1/320
Sandwich ELISA	2	8	7	7	8	10
Competitive ELISA	10	12	7	6	3	4

Anti- HDV assay for 42 anti- HDV positive serial sera

For the double antigen sandwich ELISA, the anti-HDV positive rate increased with the dilution ratio, while the anti- HDV positive rate decreased for the competitive ELISA, this results revealed a statistical association (t=2.44, $P<0.01$) between the two assays. (Table 1).

Neutralization assay

In the neutralization inhibitory test by purified HDAg, *OD* values of the 2 anti-HDV positive serial sera declined in an obvious manner, this showed that the activity of anti-HDV was significantly declined after combining with HDAg; the inhibitory rates were all more than 50%. In this assay, the *OD* values of anti-HDV positive control and anti-HDV negative control were 0.993 and 0.067 respectively (Table 2).

HDV RNA assay

Of the 42 anti-HDV positive sera, 20 (47.6%) were HDV-RNA positive; this result suggests that there was clear correlation between anti-HDV and HDV-RNA.

Specificity analysis

In this assay, negative controls of anti-HDV kit purchased from Noctech Ltd., Dublin, Ireland were measured and the OD values were all below 0.10, then the 2.1N value of this assay was assumed to be 0.21. Based on this data, 2 positive controls of anti-HDV positive sera were anti-HDV positive, all 60 samples infected by other hepatitis viruses and 30 normal samples were negative for anti-HDV.

DISCUSSION

HDAg is the only viral protein known to be expressed during HDV infection; previous studies have shown that HDAg is a phospho-protein coded by the fifth open reading frame (ORF5) of the anti-genome of HDV (8). There were 57 amino acids which have HDV antigenicity in its N-terminal region. Tanghad obtained two HDV gene fragments of 653 and 220 bp coding for polypeptides with length of 214 and 74 amino acids respectively both of which had good HDV antigenicity and immunocompetence (14). The size of the expression vector HD-PQE_{31} constructed by us was between these two fragments sizes and should be correspond to 111 amino acids. It also had a excellent HDV antigenicity and immunocompetence characteristics.

The highly efficient expression vector PQE31, which

Table 2. The results of the neutralization assay[a]

Sample		Dilution		
		1/100	1/300	1/900
1	Before neutralization (*OD*)	1.112	1.005	0.985
	After neutralization (*OD*)	0.249	0.087	0.073
	Inhibitory rates (%)	78	91	93
2	Before neutralization (OD)	1.151	0.832	0.708
	After neutralization (*OD*)	0.291	0.062	0.063
	Inhibitory rates (%)	75	93	91

[a] Rate of inhibition (%) = (Before neutralization (OD)－After neutralization (OD))/ Before neutralization (OD) ×100％.

contains the expressed HDAg protein, has 6 consecutive histidine residues which bind to the metal Ni atoms in the Ni-NTA column. In addition, we gradually decreased the concentration of urea contained in dialysis solution to dialyze urea and reverse the denaturalization of the HDAg protein. Tests of specificity, sensitivity and repetition for the HDAg protein showed that a HDAg protein with stable antigenicity could be prepared by gene engineering technology in accordance with the results according to Jiang (7). Obtaining high pure and high titer recombinant HDAg is an important basis for establishing an ELISA assay of anti-HDV in serum.

Detection of anti-HDV in serum is able to confirm HDV infection, and is of diagnostic value, especially when the titer of anti-HDV is about 1/1000 (9). We detected anti-HD in serum by competitive ELISA - in the past the positive results were determined by reverse inhibitory rates. Unlike to observations by eye, it was possible to neglect the weakly positive specimens; the sandwich ELISA could judge the weak positive specimen by difference in light color. Within the 42 anti-HDV positive serial sera, the sandwich ELISA was significantly more likely than competitive ELISA to test positive for anti-HDV, namely, when there is 'hook' effect, the sample was diluted at higher dilution and the sandwich ELISA would have a higher positive rate, Huang (5) had similar results; the results showed that the sensitivity of sandwich ELISA was higher than those of competitive ELISA (t=2.44, $P<$ 0.01).

In the selection of conditions for establishing sandwich ELISA, as a coated protein, the purified HDAg had good effects showing strong affinity to anti-HDV in human serum; we assumed that there were different antigenic determinants against anti-HDV in human serum for the purified HDAg and HDAg (pD280). Results of specific assay and neutralization assay indicated that this sandwich ELISA was inactive for antibodies against other hepatitis viruses, suggesting that it has higher sensitivity and was reliable to test anti-HDV in human serum.

We have detected 42 anti-HDV positive sera with HDV-RNA and anti-HDV assays with a positive rate of HDV-RNA of 47.6%. HDV-RNA was generally located in liver cells and detection of HDV-RNA was confirmed by RT-PCR and complementarity to serological markers for HDV infection (10), but there were some discrepancies in results obtained with assays for HDV-RNA and anti-HDV in serum.

Considering that hepatitis B infection prevalence is high in China and HDV not only influences the prognosis but also the therapeutic response, HDV superinfection in a chronic HBV carrier often leads to a significant morbidity and mortality. Acute HDV and HBV co-infection is frequently associated with fulminant hepatitis. Serum ALT levels in anti-HDV-positive HBsAg carriers were significantly higher than those without HDV RNA and liver damage in these patients may be caused mainly by ongoing HDV infection not by HBV replication (11). Whereas anti-HDV may be undetectable during the acute phase of hepatitis, this means that the prevalence of HBV/HDV coinfection is sometimes underestimated during the acute phase of disease (12). Combined detection of HDV-RNA, HDAg and anti-HDV in sera will have a practical etiologic diagnostic value, in particular, an assay for anti-HDV has significance for past infection, epidemiological studies, and chronic infection (13). In our study, we established a new sandwich ELISA for

detecting anti-HDV in human serum which had higher specificity and sensitivity without needing special equipment and required a shorter testing time. This suggests that the new sandwich ELISA will be useful in routine laboratories for diagnosis and monitoring of HDV infection and a valuable alternative to epidemiological research of HDV infection in developing countries.

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