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Development of a Hepatitis Delta Virus Antibody Assay for Study of the Prevalence of HDV Among Individuals Infected with Hepatitis B Virus in China

Liping Shen,[1] Ying Gu,[2] Le Sun,[3] Yingchao Yang,[1] Feng Wang,[1] Yimin Li,[1] and Shengli Bi[1]*

[1]*State Key Laboratory for Molecular Virology and Genetic Engineering, National Institute for Viral Disease Control and Prevention, Chinese Center for Disease Control and Prevention, Changping District, Beijing, China*
[2]*National Institute of Diagnostics and Vaccine Development in Infectious Diseases, School of Life Sciences, Xiamen University, Xiamen, Fujian, China*
[3]*AbMax Biotechnology Co. Ltd, Haidian District, Beijing, China*

Co-infection with hepatitis delta virus (HDV) and hepatitis B virus (HBV) has been shown to be associated with a more severe form of acute and chronic hepatitis. Cloning and expression of recombinant HDV antigen (rHDAg) in *Escherichiacoli* are described. Using purified rHDAg, a cost-effective indirect anti-HDV enzyme-linked immunosorbent assay (ELISA) kit was developed. Direct comparison of 15 known HDV-positive sera and 15 HDV-negative sera showed concordance agreement between the new assay kit and the Abbott Murex Anti-Delta (total) kit. In addition, 1,486 hepatitis B surface antigen (HBsAg) positive blood samples collected from various areas of China were tested using this indirect anti-HDV ELISA. It was found that 1.2% (95% CI: 0.7–1.9%) of the samples were anti-HDAg positive. It is suggested that the prevalence of HDV and HBV co-infection in China is relatively low. ***J. Med. Virol. 84:445–449, 2012.***

KEY WORDS: hepatitis delta virus; antigen; antibody; enzyme-linked immunosorbent assay; prevalence

INTRODUCTION

Hepatitis delta virus (HDV) is a defective single-stranded RNA virus that requires hepatitis B virus (HBV) co-infection to complete its life cycle [Rizzetto et al., 1977; Rizzetto et al., 1980]. HDV contains a ribonucleoprotein core, a circular single-stranded RNA genome approximately 1.7 kb long, and 70–200 copies of the delta antigen gene per RNA molecule [Wang et al., 1986; Ryu et al., 1992]. The study of the replication of HDV in hepatocytes has shown that the hepatitis B surface antigen (HBsAg) is an essential helper. Co-infection with HBV and HDV has been shown to be associated with a more severe form of acute and chronic hepatitis in many HBsAg-positive individuals compared to those with HBV infection alone [Wu et al., 1991; Taylor, 2003]. This more severe form of hepatitis results in a faster advance to cirrhosis, fulminant hepatitis, and hepatocellular carcinoma [Jacobson et al., 1985; Govindarajan et al., 1993; Gupta et al., 2005; Zaidi et al., 2010].

HDV can be identified both in liver biopsies and in patient sera using assays that detect HDAg or anti-HDAg. Serological studies of HDV infection in HBsAg-positive individuals have shown that hepatitis D infection is distributed worldwide [Radjef et al., 2004; Mumtaz et al., 2005; Abbas et al., 2010; Bahcecioglu et al., 2011]. Endemic HBV is a serious health problem in China, and 7.18% of the total population are HBsAg carriers [Liang et al., 2009]. In the 1990s, several studies by different investigation reported that the prevalence of HDV based on sera ranged from 0.8–12% in different provinces of China [Wang et al., 1987; Mai, 1989; Chen et al., 1990, 1998; Hao et al., 1990; Zhao, 1990; Zhang et al., 1995]. However, only limited HDV infection have been documented in the large number of persons infected with HBV in

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Liping Shen and Ying Gu contributed equally to this work.

*Correspondence to: Shengli Bi, MA, State Key Laboratory for Molecular Virology and Genetic Engineering, National Institute for Viral Disease Control and Prevention, Chinese Center for Disease Control and Prevention, 155# Changbai Road, Changping District, Beijing 102206, China. E-mail: shengli_bi@163.com

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China. The main reasons are the high cost of imported anti-HDAg diagnosis reagents, such as those produced by Abbott (Chicago, IL) or Adaltis (Montreal, Canada), and the low sensitivity and specificity of anti-HDAg diagnostic kits produced domestically.

In China, the HDAg used in diagnostic kit was extracted from the sera of patients infected with HDV before technique of genetic engineering had been applied. The source of sera is very limited. The development of a high-quality anti-HDAg diagnostic reagent requires a recombinant HDAg protein with high purity and high antigenic activity.

In this study, HDAg cDNA was cloned from a strain of HDV isolated from a patient in Inner Mongolia, China. The soluble rHDAg expressed in *Escherichia coli* showed excellent antigenic activity. An indirect anti-HDAg enzyme-linked immunosorbent assay (ELISA) was developed successfully using this protein. A study involving 1,486 HBsAg-positive samples was carried out to determine the prevalence of HDV and HBV co-infection in China.

MATERIALS AND METHODS

Cloning and Expression of HDAg

HDV RNA was isolated from blood samples collected from Inner Mongolia in 2005 by using the QIAamp UltraSens™ Virus Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The extracted RNA (10 μl) was reverse transcribed into cDNA with SuperScript™ II Reverse Transcriptase (Invitrogen, Carlsbad; CA). A nested polymerase chain reaction (PCR) was carried out using the following primers, which were designed based on the published HDV Ag sequences (GeneBank accession number is X77627): PM1 (5′GCG GCA TAI GAT GAG CCG CT-3′), PM2 (5′-TAG TCT CGA GCT GGG GTC GAC AAC-3′), modified PM1 (5′-GCG GCA TAT GAT GCA CCA TCA TCA TCA TCA TAG CCG CT-3′), and modified PM2 (5′-TAG TGT CGA CCT GGG GAC GGC AAC T-3′). The PCR fragments were purified by using the Wizard SV Gel and PCR Clean-Up System (Promega, Madison, WI) according to manufacturer's recommendations. Purified PCR product was ligated into the pMD18-T vector (Takara, Otsu, Japan) at 16°C for 4 hr and then transformed into DH5α competent cells. The cells were spread on an agar plate, and single colonies were picked to identify the plasmids containing the target DNA fragment by enzymatic digestion. The sequencing of the recombinant plasmid was performed by Sunbiotech Co. (Beijing, China).

The pMD18-T plasmids containing the target DNA fragment were digested with *Nde* I and *Xho* I. The target DNA fragment was purified and legated into the pET-43a vector (Novagen, Madison, WI) that had been pre-digested by *Nde* I and *Sal* I, and the ligated vector was transformed into *Escherichia coli* BL21 Rosetta competent cells (Novagen). Single colonies were picked for sequencing.

The expression of recombinant proteins was induced by the addition of 1.0 mM IPTG for 2 hr. The molecular weights and expression levels of rHDAg from different bacterial clones were analyzed using 13.5% SDS–PAGE. For large-scale culture, 0.15 ml of bacteria was used to seed 300 ml luria–bertani broth. The culture was incubated at 37°C overnight and then at 32°C for 1.5 hr. When the bacterial culture OD_{600} reached 0.5, IPTG was added to a final concentration of 1.0 mM and the incubation was continued for a further 4 hr. The bacteria were harvested by centrifugation at 4,000 rpm at room temperature for 10 min. The cells were re-suspended in lysis buffer (500 mM NaCl, 20 mM Tris-HCl, 10% glycerol, and 0.1% Tween 20), sonicated on ice 40 times, and centrifuged at 12,000 rpm at room temperature for 10 min. The dissolved proteins were precipitated using saturated NH_4SO_4, and HIS-tagged rHDAg was purified using an Ni affinity chromatography column.

Blood Sample Collection

Blood samples were collected by venepuncture. Serum was prepared and stored at −20°C before testing. The survey protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the Chinese CDC Ethics Committee, and the study was in accordance with national ethics regulations. Participants were informed of the study purpose and their right to maintain information confidential. A total of 15 negative blood samples and 15 HDV-positive blood samples (screened by reverse-transcriptase PCR) were collected from different district hospitals in Beijing, China from 2008 to 2009. These samples were used to identify the antigenic activity of rHDAg and to confirm the results of the indirect ELISA using the Abbott Murex Anti-Delta (total) kit (Abbott, Chicago, IL). To study HDV prevalence in China, 1,486 serum samples were collected from the national HBV infection survey that was carried out in different areas of China between 2006 and 2008. These serum samples were shown to be HBsAg-positive using the Abbott Murex ELISA system.

Analysis of Activity of rHDAg and Comparison With Abbott Murex Anti-Delta Assay

For indirect anti-HDAg ELISA, various amounts of purified rHDAg were diluted in 0.05 M sodium bicarbonate buffer and coated on the wells of 96-well plates at 37°C for 2 hr. The coated plates were washed three times with sodium bicarbonate buffer and blocked with 3% bovine serum albumin in phosphate buffered saline. Each well then received 50 μl serum sample and was incubated for 1 hr at 37°C. After washing, a horseradish peroxidase (HRP)-conjugated goat anti-human IgG secondary antibody was added, and the plates were incubated for 1 hr at 37°C. After

five washes with phosphate buffered saline with Tween 20, HRP substrate 3,3′,5,5′-Tetramethylbenzidine solution was added, and A_{450} was determined after 15 min using a microplate reader. In the present ELISA, a specimen is considered as positive whenever the S/CO value is $\geq$1.0. The cut-off value is the average value of the negative control multiplied by 2.1. Assays using the Abbott Murex anti-Delta (total) Kit were carried out according to the manufacturer's instructions. A sample was considered positive whenever the S/CO value was <0.8 or negative whenever the S/CO value was >1.2. The cut-off value was defined as the sum of A_{450} value of the positive control and the negative control divided by 2.

RESULTS

Cloning and Expression of HDAg

Total RNA was isolated from a blood sample from patient infected with HDV from Inner Mongolia, China, and a cDNA library of HDV was constructed. A nested PCR was carried out using primers designed based on the published HDAg sequences (GeneBank accession number is X77627). A 600-bp PCR product was obtained. After sequencing, it was found to be concordance identical to the reported HDV RNA Ag gene sequence in the GenBank (data not shown).

The HDAg cDNA fragment was ligated into the pET-43a expression vector, which was then transformed into *E. coli* BL21 Rosetta competent cells. Over 20 colonies were picked and tested for rHDAg expression after IPTG induction. The recombinant protein with the expected molecular weight of ~27 kDa was induced and accounted for approximately 20% of the total bacteria protein (Fig. 1, lane 3). The rHDAg protein was found in the supernatant of the cell lysates. After purification by precipitation with saturated NH_4SO_4, followed by chelating affinity chromatography, the purity of rHDAg reached 95%, as shown in lanes 4 and 5.

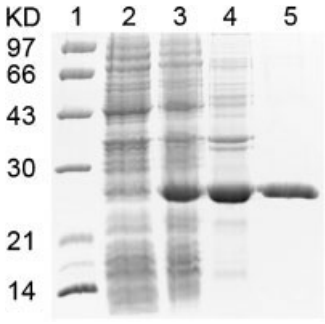

Fig. 1. SDS–PAGE analysis of rHDAg expression. lane 1, protein molecular weight markers; lane 2, negative control BL21 cells; lane 3, transformed BL21 cells that expressed rHDAg protein; lane 4, rHDAg protein after precipitation with saturated NH_4SO_4; and lane 5, purified rHDAg after the chelating column.

TABLE I. Antigenic Activity of rHDAg

Sample No.	A_{450} values of different dilutions of rHDAg			
	100 ng/well	50 ng/well	10 ng/well	5 ng/well
P1	2.78	2.34	1.76	0.089
P2	2.35	2.11	1.39	0.087
P3	2.09	1.87	1.401	0.086
P4	1.98	1.91	1.03	0.076
P5	2.17	1.86	1.43	0.085
N1	0.106	0.086	0.058	0.056
N2	0.077	0.072	0.053	0.054
N3	0.092	0.065	0.065	0.063
N4	0.064	0.056	0.058	0.052
N5	0.066	0.069	0.060	0.067

P, HDV-positive sera; N, normal sera.

rHDAg Retains Antigenic Activity for Detection of Anti-HDAg in Blood Samples From HDV-infected Patients

To test the antigenic activity of the recombinant HDV Ag, various amounts of purified rHDAg were coated on 96-well plates, and an indirect ELISA was carried out. Sera from five patients infected with HDV and five negative controls were used as the primary antibodies. With the coating of rHDAg between 10 and 100 ng/well, all the five HDV-positive sera had an OD >1.0, whereas all five negative controls had the same reading as the background (Table I). When the coating of rHDAg was 5 ng/well, all the 10 sera had readings equivalent to background. These data show that the rHDAg retained excellent antigenic activity for the detection of anti-HDV antibodies in HDV-infected samples. An indirect anti-HDAg ELISA was developed using 50 ng/well of rHDAg.

Comparison of Indirect Anti-HDAg ELISA With Abbott Murex Anti-Delta (Total) Kit

To validate the indirect anti-HDAg ELISA, a direct comparison test using the Abbott Murex Anti-Delta (total) kit was carried out. There were 15 serum samples with HDV and 15 control sera. The data are shown in Table II. The kit of Abbott is a competitive inhibition ELISA, and a specimen is considered positive whenever the S/CO value is lower than the cut-off value, which was 0.8905, defined as the sum of the A_{450} values of the positive control and the negative control divided by 2. In the indirect anti-HDAg ELISA, a specimen is considered positive whenever the S/CO value is $\geq$ 1, and the cut-off value is defined as the average values of the negative controls multiplied by 2.1. It is shown that the results of 15 serum samples with HDV are anti-HDAg-positive, and results of 15 control serums are anti-HDAg-negative using two assays, indicating complete agreement between the test results of the two assays. Therefore, the new assay could be used for screening for HDV infection.

TABLE II. Comparison of the Indirect ELISA and the Abbott Murex anti-Delta (total) ELISA

Sample No.	Abbott Murex (S/CO value)	Indirect ELISA assay (S/CO value)
P1	0.081	8.26
P2	0.069	3.51
P3	0.075	16.06
P4	0.076	24.04
P5	0.079	10.09
P6	0.108	4.16
P7	0.074	24.62
P8	0.075	16.95
P9	0.094	12.74
P10	0.149	10.46
P11	0.074	6.70
P12	0.077	5.58
P13	0.091	16.23
P14	0.067	3.16
P15	0.078	6.36
N1	2.735	0.81
N2	2.011	0.83
N3	1.941	0.94
N4	2.242	0.70
N5	1.892	0.91
N6	2.342	0.77
N7	2.014	0.98
N8	1.982	0.56
N9	1.852	0.67
N10	1.769	0.80
N11	1.986	0.71
N12	1.586	0.90
N13	1.764	0.78
N14	2.014	0.52
N15	2.130	0.74

The cut-off value of the Abbott assay was 0.8945. The cut-off value of the indirect ELISA assay was 0.069.

Prevalence of HDV/HBV Co-Infection in China

To study the prevalence of HDV/HBV co-infection in China, 1,486 samples with HBsAg were collected from five different areas in China and were tested using the new indirect anti-HDAg ELISA. Only 18 out of the 1,486 samples were anti-HDAg positive, indicating an HDV prevalence of 1.2% among persons infected with HBV (Table III). Comparing the numbers from different areas, it appears that the rate of HDV/HBV co-infection in Southwest China is slightly higher than other areas of China ($\chi^2 = 3.814$, $P > 0.05$).

TABLE III. Distributions of HDV prevalence in China by region

Area of China	Sample size	anti-HDAg positive		
		Number	Rate	95% CI
Eastern China	275	2	0.73	0.09–2.60
Western China	312	2	0.64	0.08–2.28
Central China	326	3	0.92	0.19–2.66
Northern China	291	4	1.37	0.37–3.46
Southern China	282	6	2.13	0.79–4.58
Total	1,486	18	1.21	0.72–1.91

Note: $\chi^2 = 3.814$, $P > 0.05$.

DISCUSSION

HDV requires HBV co-infection to complete its life cycle; 7.18% of the total population are HBV carriers in China [Liang et al., 2009]. Previously, due to the lack of a cost-effective HDV assay, studies were restricted to using a relatively small number of samples, which resulted in a varied prevalence of HDV from 0.8 to >12% in different provinces of China [Wang et al., 1987; Mai, 1989; Chen et al., 1990, 1998; Hao et al., 1990; Zhao, 1990; Zhang et al., 1995].

A reliable and low-cost indirect anti-HDAg ELISA was developed based on *E. coli*-expressed recombinant HDV Ag. Using this kit, a study of 1,486 HBV-positive samples from different areas of China was carried out. It was found that only 1.2% of the individuals infected with HBV were co-infected with HDV. HDV prevalence in the individuals infected with HBV varies in different areas of the world, ranging from low prevalence in Switzerland (<6%) to 30% in regions such as Moldavia, Romania, Southern Italy, and other Mediterranean countries [Grabowski and Wedemeyer, 2010; Genne and Rossi, 2011]. It is suggested that the prevalence of HDV infection in China is relatively low. A study using a much larger number of patients with HBV infection will be carried out.

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