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RAPID COMMUNICATION

Significance of serum IgA in patients with acute hepatitis E virus infection

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Abstract

AIM: To study the significance of serum anti-hepatitis E virus (HEV) IgA in patients with hepatitis E.

METHODS: A new method was established to assay anti-HEV IgA, which could be detected in the middle phase of the infection. We compared anti-HEV IgA assay with anti-HEV IgM and anti-HEV IgG assay in sera from 60 patients with positive HEV-RNA.

RESULTS: The 60 patients with positive HEV-RNA had both anti-HEV IgA and anti-HEV IgM and 410 patients with negative HEV-RNA were used as control. Periodic serum samples obtained from 60 patients with hepatitis E were tested for HEV RNA, anti-HEV IgM, anti-HEV IgA and anti-HEV IgG. Their HEV-RNA was detectable in the serum until 20 ± 11 d. We used anti-HEV IgM and anti-HEV IgA assay to detect HEV infection and positive results were found in 90 ± 15 d and 120 ± 23 d respectively, the positive rate of anti-HEV IgA was higher than that of anti-HEV IgM and HEV-RNA (P <0.05).

CONCLUSION: The duration of anti-HEV IgA in serum is longer than that of anti-HEV IgM, and anti-HEV IgA assay is a good method to detect HEV infection.

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Key words: Hepatitis E virus; RNA; Anti-HEV IgM; Anti-HEV IgA

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INTRODUCTION

Hepatitis E is caused by infection with hepatitis E virus (HEV), which is a non-enveloped single-stranded RNA virus. Just like hepatitis A virus (HAV), HEV is usually transmitted from person-to-person via the fecal-oral route. Epidemic of hepatitis E has been found in Central and South-East Asia, North and West Africa, and Mexico, where fecal contamination of drinking water is common. However, recent reports showed that HEV occurs in individuals without traveling in HEV epidemic areas in developed countries like the United States of America and Japan[1-5]. The application of recently developed serologic tests has revealed anti-HEV in almost all countries, including developed countries, in which the disease virtually does not occur[6-8]. Possible reservoirs of HEV in the developed countries may be found in animals like monkeys, pigs, cows, rodents, sheep or goats, suggesting that HEV may be zoonotic[9-12]. Xia *et al*[13] reported that acute hepatitis E occurs in rhesus monkeys, indicating that transfusion transmits hepatitis E. Hepatitis-E is a self-limited disease. But a current research indicates that there are cis-reactive elements in HEV ORF3[14]. Surjit M *et al*[15] found that enhanced alpha microglobulin secretion from Hepatitis E virus ORF3-expressing human hepatoma cells is mediated by the tumor susceptible gene 101. HEV is highly epidemic in China and it is very important for us to find a reliable assay for diagnosing its acute, sub-clinical and chronic infection.

Immunoglobulin M (IgM) against HEV (anti-HEV IgM) is used as a reliable and sensitive marker for recent HEV infection[16-19]. However, the specificity of solid-phase assay for anti-HEV IgM has been questioned in patients with IgM-rheumatoid factors in serum. It was reported that anti-HEV IgA can be utilized as an additional complementary antibody for detecting recent HEV infection[20-23]. But the clinical and epidemiological implications of anti-HEV IgA in finding HEV infection remain to be clarified.

In this study, we used the solid-phase assay to detect HEV-specific IgA. The kinetics of anti-HEV IgM, anti-HEV IgA and anti-HEV IgG responses in serum from patients with hepatitis E demonstrated that anti-HEV IgA assay could identify HEV infection.

MATERIALS AND METHODS

Serum samples

This study enrolled 100 patients (77 males and 23 females,

mean age 52 ± 11 years) with acute hepatitis E admitted to the Department of Infectious Diseases of Tongji Hospital in Wuhan, China, during the period of August 2003 - August 2004. Sera were collected from 60 HEV-RNA positive patients on d 15, 30, 60, 90 and 120 after the onset of symptoms. In addition, 410 serum samples obtained from HEV-RNA negative patients (304 males and 106 females, mean age 42 ± 9 years) were used as controls, including 100 samples from voluntary blood donors with normal alanine aminotransferase (ALT) level, 218 samples from patients with hepatitis A, B or C, 52 samples from patients with autoimmune hepatitis, 40 samples from patients with rheumatoid arthritis. Liver function tests included total serum bilirubin (T-Bil) and direct bilirubin (D-Bil, aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase, total protein, γ-glutamyltranspeptidase, prothrombin time, and total cholesterol levels. The serum samples were stored at -70℃ until HEV RNA test.

The presence of anti-HAV IgM, hepatitis B surface antigen (HbsAg), hepatitis B e antigen (HbeAg), antibodies to HBV core (anti-Hbc), and anti-HCV was determined with commercially available kits.

RNA extraction and RT-PCR

Total RNA was extracted with guanidinium isothiocyanate. RT-PCR was carried out in a 50 μL reaction volume containing 10 μL purified RNA, 25 pmol each primer, 1 μL AMV reverse transcriptase and 0.5 μL Taq polymerase. Thirty-five cycles of PCR amplification were performed at 94℃ for 1 min, at 52℃ for 45 s, at 72℃ for 1 min, and a final extension at 72℃ for 7 min. Second-round reactions were carried out in a volume of 50 μL containing 10 μL first-round product, 25 pmol each primer and 0.5 μL Taq polymerase. Thirty PCR omplification cycles were performed at 94℃ for 1 min, at 52℃ for 45 s, at 72℃ for 1 min, and a final extension at 72℃ for 7 min.

Consensus oligonucleotide primers for ORF2 were designed within regions of conserved sequences in the full-length sequences from Asia, Mexico, USA and China strains. The ORF2 primers, at positions 5681nt-6486nt, produced a product of 805 bp nucleotide: EP1 (5´-GCTTCAAATTATGCCCAGTA-3') and EP2 (5' -TGTTGGTTGTCATAATCCTG-3'). Second round internal primers amplified 345 bp products for ORF2: EP3 (5´-GTTATGCTTTGCATA(T)CATGG-3') and EP4 (5' -CCGACGAAATCAATTCTGTC- 3').

Sequence analysis

Second-round PCR products were purified and ligated into pMD 18-T vectors. Twenty-five clones were sequenced automatically with an ABI model - 3730 DNA sequencer and the ABI sequencing ready reaction kit (Perkin - Elmer) according to the manufacturer's instructions. Sequences used for phylogenetic analysis were obtained from Mexico (GenBank accession no. M74506), Burma (D10330), USA (AF060669), China (C1, AJ272108; C2, AF103940; C3, AF151963).

Phylogenetic analysis

Nucleotide sequences were aligned using Clustalx 1.81 (http://downloads.scalableinformatics.com/downloads/clustalx/). These alignments were analyzed with Mega 2.0 (http://evolution.genetics.washington.edu/phylip/software.html) to evaluate the evolutionary distances between sequences using 1000 bootstraps of the data. Bootstrap values greater than 70% could provide significant evidence for phylogenetic analysis grouping.

ELISA for anti-HEV-IgM and anti-HEV-IgG

Anti-HEV-IgM and anti-HEV-IgG were detected by using commercially available kits (BeiJing Wantai Biological Pharmacy Enterprise Co., LTD.). Absorbance (*A*) value was measured at 450 nm. Anti-HEV-IgM cut off = 0.26 + negative control mean *A* value, Anti-HEV-IgG cut off = 0.16 + negative control mean *A* value. Samples with *A* values > cut off were considered positive, Samples with *A* values ≤ cut off were considered negative.

ELISA for anti-HEV-IgA

ELISA for detection of IgG using purified recombinant ORF2/ORF3 protein (WanTai biological pharmacy enterprise co. LTD.) was performed with HRP-labeled coat monoclonal anti-human IgA (Wuhan Lingfei Science and Technology Limited Company) in stead of HRP-labeled coat monoclonal anti-human IgG. In order to detect the titers of goat anti-human IgA-HRP, ELISA was performed with 6 diluted concentrations: 1:500, 1:1000, 1:2000, 1:4000, 1:8000, 1:16000. Results demonstrated that P/N (ratio of sample *A* to negative *A*) of 1:4000-1:8000 of diluted antibodies was ≥ 2.0. In order to obtain the stably detectable results, the diluted concentration of 1:4000 was adopted as standard one and 410 sera with negative HEV-IgM and HEV–IgG were detected for HEV-IgA.

Statistical analysis

Chi square test was used for statistical analysis.

RESULTS

Among the 100 acute phase sera with hepatitis E, 60 (60%) were positive for HEV RNA by reverse transcription- nested polymerase chain reaction (RT- nPCR) using the degenerated primers. Among which 25 partial ORF2 sequences (5972-6317 nt) belonging to HEV genotype Ⅳ were analyzed.

HEV RNA in serum samples from 100 donors negative for anti-HEV-IgM and anti-HEV-IgG was detected by anti-HEV-IgA assay. The absorbance (A) was set at 0.128, standard deviation (SD) at 0.053. When critical value was set at A + 2SD, the IgA assay results were all negative in 410 HEV-IgM and -IgG negative sera. When the A value of positive HEV-IgA assay was set as the mean value of negative control plus 0.18, the specificity of anti-HEV IgA was verified by absorption with the same recombinant ORF2 protein (50 mg/L at the final concentration for anti-HEV IgA assay). Briefly, prior to assay, 10 serum samples were diluted at 1:100, 1:500, 1:1000, 1:5000 to make its A value < 1.5. The microwells were incubated at 37℃ for 0.5 h, then at 4℃ overnight. When the ten serum samples were diluted at 1:500, the A value of the tested samples

Table 1 Prevalence of anti-HEV IgM and anti-HEV IgA in different groups of subjects

Group	*n*	Age (mean ± SD)	Anti-HEV IgA, *n* (%)	Anti-HE IgM, *n* (%)	Anti-HEV IgG, *n* (%)	HEV RNA, *n* (%)
Hepatitis A	62	33.1 ± 11.2	2 (3.2)	1 (1.6)	1 (1.6)	0
Hepatitis B	100	36.3 ± 12.2	1 (1)	0	10 (10)	0
Hepatitis C	56	45.6 ± 15.3	1 (1.8)	1 (1.8)	1 (1.8)	0
Autoimmune hepatitis	52	40.5 ± 10.1	0	2 (3.8)	3 (5.8)	0
Rheumatoid arthritis	40	56.4 ± 9.4	0	2 (5)	1 (2.5)	0
Blood donors	100	36.8 ± 18.5	2 (2)	1 (1)	8 (8)	1 (1)
Hepatitis E	100	52.6 ± 14.8	100 (100)	100 (100)	98 (98)	60 (60)

Table 2 Serum anti-HEV IgA, anti-HEV IgM, HEV RNA, ALT and TBil

T/mo	HEV RNA *n* (%)	Anti-HEV -IgA *n* (%)	Anti-HEV -IgM *n* (%)	Anti-HEV -IgG *n* (%)	ALT (mean ± SD) nkat/L	TBil (mean ± SD) μmol/L
-0.5	60 (100)	60 (100)	60 (100)	59 (98)	1117 ± 200	86 ± 17
-1	36 (60)	60 (100)	60 (100)	60 (100)	4101 ± 884	506 ± 123
-2	3 (5)	58 (97)[a]	46 (77)	60 (100)	1850 ± 750	198 ± 68
-3	0	56 (93)[b]	34 (57)	60 (100)	800 ± 250	84 ± 31
-4	0	38 (3)[b]	12 (20)	60 (100)	567 ± 183	46 ± 14
-5	0	18 (0)[b]	2 (3)	60 (100)	467 ± 150	23 ± 8

[a]$P < 0.05$, [b]$P < 0.01$ compared with anti-HEV IgM positive results.

was reduced by ⩾ 75% in the anti-HEV IgA assay with the recombinant ORF2 protein, the sample was considered positive for anti-HEV.

Among the serum samples obtained from 100 blood donors, only one sample was positive for anti-HEV IgM, and two samples were positive for anti-HEV IgA. The same samples were positive for anti-HEV IgG and HEV RNA. Using the cutoff values, among remaining 310 serum samples obtained from 62 patients with hepatitis A, 100 patients with hepatitis B, 56 patients with hepatitis C, 52 patients with autoimmune hepatitis, 40 patients with rheumatoid arthritis, were tested for anti-HEV IgA, anti-HEV IgM and anti-HEV IgG (Table 1). Among the 410 patients who were not infected with HEV, including the 100 blood donors, anti-HEV IgM was positive in 7 serum samples, including 2 from patients with hepatitis, 4 from patients with autoimmune disorders. Anti-HEV IgA was detected in 6 serum samples including 4 from patients with hepatitis. The positivity for HEV antibodies could not be confirmed by the absorption test in any of the 10 samples. Serum samples obtained from 100 patients with sporadic acute hepatitis E were tested for the presence of IgM and IgA anti-HEV antibodies. All the 100 patients were positive for anti-HEV IgM and anti-HEV IgA. The presence of anti-HEV IgM and anti-HEV IgA was confirmed by the absorption test in the serum samples from all 100 patients. HEV IgA assay was highly specific and false- positive results in HEV-IgG and IgM assay could also be found in the IgA assay (Table 1).

Anti-HEV IgA, IgM and HEV-RNA were positive in patients with early HEV infection. HEV RNA remained detectable in the serum for one month, anti-HEV IgA for four months (> 50%, anti-HEV IgM for three months (> 50%), ALT and T-Bil for four months (Table 2).

DISCUSSION

Although the incidence of acute hepatitis especially acute hepatitis E has been gradually increased, the correct diagnosis of HEV infection largely depends on the sensitivity and specificity of HEV assay kits. The golden diagnosis standard for acute hepatitis E is based on detection of the HEV genome in serum or feces by RT-PCR, but it can only be used for earlier diagnosis. A solid-phase (sandwich or indirect) ELISA for detecting anti-HEV IgM and IgG is simple and has been currently used in diagnosis of HEV infection[24]. One of its disadvantages is the reduced sensitivity due to competition among virus-specific IgM, IgA and IgG for antigen-binding sites. Another potential disadvantage of the solid-phase test for IgM antibody is that IgM-rheumatoid factor in sera from patients with rheumatoid arthritis may produce a false-positive result. Although the existence of anti-HEV IgG in serum is very long, we cannot distinguish current acute infection from the last infection. It is important to find a new diagnosis assay. It has been reported that anti-HEV IgA can be utilized as an additional confirmatory antibody for recent HEV infection[25]. Although the presence of a specific antibody of IgA can show recent infection in several viral or nonviral diseases, including hepatitis A and B as well as cholera[26], the clinical and epidemiological significance of positivity for anti-HEV IgA remains to be fully verified.

In the present study, we used ELISA to detect IgA using HRP-labeled coat monoclonal anti-human IgA in stead of HRP-labeled coat monoclonal anti-human IgG. The results showed both anti-HEV IgM and anti-HEV IgA were detectable in serum samples obtained from all 60 patients tested who were subsequently diagnosed with HEV infection. The sensitivity was 100%. Among the 410 serum samples from subjects not recently infected with HEV, 6 were falsely positive for anti-HEV IgM and 5 were falsely positive for anti-HEV IgA, indicating that the false-positive rate is low both in anti-HEV IgM and in anti-HEV IgA. Except for one occult HEV infection, none was positive for both anti-HEV IgM and anti-HEV IgA. The specificity of anti-HEV IgA was verified in the absorbant and inhibitory assay.

HEV RNA, anti-HEV IgA, anti-HEV IgM, ALT and TBiL were defected in serum samples from 60 infected patients during follow-up. From these 60 patients, the serum samples obtained on d 15, 30, 60, 90 and 120 after the disease onset. The period of apparent manifestations was 40 d, liver disfunction usually lasted 5 mo, the peak of alanine aminotransferase and total bilirubin was 246 ± 53 U/L and 506 ± 123 μmol/L respectly. The positive number of HEV RNAs in serum was 60 (100%), 36 (60%), 3 (5%) and 0 in wk 2, mo 1-3 respectly. The positive number of anti-HEV IgA was 60 (100%), 60 (100%), 58 (97%), 56 (93%), 38 (63%), 18 (30%) in the second week, the first

1-5 mo after the disease onset, respectively. The positive number of anti-HEV IgM was 60 (100%), 60 (100%), 46 (77%), 34 (57%), 12 (20%), 2 (3%) in the second week, the first 1-3 mo, respectively. The positive rate of anti-HEV IgM and anti-HEV IgA was not significantly different in the first month after the disease onset. From the second month, the positive rate of anti-HEV IgM decreased sharply. The positive rate of anti-HEV IgA maintained at a high level. There were significant changes in them ($P < 0.05$). Since the duration of sero-positivity for HEV RNA, anti-HEV IgM and anti-HEV IgA lasted 1, 3 and 4 mo respectively, hepatic dysfunction lasted 4 mo, the diagnosis of HEV infection could be made by assay of anti-HEV IgM and anti-HEV IgA. Anti-HEV IgA and anti-HEV IgG can be used as a diagnostic indicator of HEV without anti-HEV IgM.

We have previously reported that indirect ELISA can detect anti-HEV IgG and anti-HEV IgA. The performance of IgA test has been extensively evaluated[27,28]. The assay specificity, sensitivity, and consistency are carefully documented. Based on the results obtained in the study, we conclude that anti-HEV IgA assay is as specific as the anti-HEV IgM assay in diagnosis of hepatitis E. IgM and IgA anti-HEV tests can be used together to characterize serum specimens with non-acute hepatitis E. Detecting both IgM anti-HEV and IgA anti-HEV at the same time help to minimize the false positive rate, thus improving diagnosis of HEV infection.

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