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Diagnostic Value of Immunoglobulin G (IgG) and IgM Anti-Hepatitis E Virus (HEV) Tests Based on HEV RNA in an Area Where Hepatitis E Is Not Endemic

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Acute hepatitis E (AHE) has rarely been reported in industrialized countries, but the rate of seroprevalence of hepatitis E virus (HEV) antibodies (anti-HEV) is inappropriately high. The sensitivity and specificity of the assay used to test for immunoglobulin G (IgG) and IgM anti-HEV have not been well established in areas where hepatitis E is not endemic (hereafter referred to as "nonendemic areas"). We collected serum samples from 13 AHE patients, 271 healthy subjects, and 160 other liver disease patients in Taiwan to test for HEV RNA by reverse transcription (RT)-PCR and for IgG and IgM anti-HEV by enzyme-linked immunosorbent assays. The sensitivities of IgG and IgM anti-HEV (relative to RT-PCR) were 86.7 and 53.3%, respectively. The specificities of IgG and IgM anti-HEV assays for diagnosing AHE were 92.1 and 98.6%, respectively. The rate of seroprevalence of IgG anti-HEV was 11% among healthy subjects in this nonendemic area, and it increased with age. In summary, IgG anti-HEV is a good diagnostic test for screening for AHE in nonendemic areas. The high rate of prevalence of anti-HEV in healthy subjects indicates that subclinical infection may exist.

Hepatitis E virus (HEV), often spread by feces-contaminated drinking water, causes a self-limiting acute hepatitis (2, 3, 29). Acute hepatitis E (AHE) has only been sporadically found in industrialized countries (18, 28); however, the rates of prevalence of antibodies to HEV (anti-HEV) are 1 to 5% in the general population (26) and as high as 21.3 to 31% in American blood donors (28). The rate of prevalence of immunoglobulin G (IgG) anti-HEV is up to 10.7% in the general population in Taiwan (17), but AHE cases are rarely found, and most of them have involved a history of travel to endemic countries (32). The reason for the discrepancy between the high rates of seroprevalence of anti-HEV in the general population and the low incidence of symptomatic AHE in these areas is not clear.

Enzyme immunoassays based on recombinant proteins of HEV have been used for most seroprevalence studies. The recombinant proteins contain immunodominant epitopes encoded by open reading frame 2 (ORF2) and ORF3 of the HEV genome from different strains (33). A wide range of sensitivity and specificity has been reported for these assays (7, 8, 10, 20, 34). This information implies that these assays might be unreliable for the diagnosis of HEV infection in areas where hepatitis E is not endemic (hereafter referred to as "nonendemic areas"). However, most anti-HEV assays have not been correlated with HEV RNA determined by reverse transcription (RT)-PCR. In this study, we evaluated the diagnostic value for AHE patients of commercial anti-HEV IgG and IgM enzyme-linked immunosorbent assays (ELISA) relative to HEV RNA detection. The prevalence of anti-HEV among the general population in Taiwan was also reevaluated with these assays.

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MATERIALS AND METHODS

AHE patients. Eleven AHE patients who had been determined to be positive for HEV RNA were included in this study. All 11 patients had serum transaminase levels 10-fold higher than the upper limit. They were negative for IgM antibody to hepatitis A virus (anti-HAV), hepatitis B virus surface antigen (HBsAg), IgM antibody to hepatitis B virus core antigen (anti-HBc), and antibody to hepatitis C virus (anti-HCV). Of the 11 patients (28 to 74 years old), 9 were men and 2 were women. All were admitted to Taipei Veterans General Hospital (Taipei VGH), a medical center in northern Taiwan, from May 1990 to July 1997. Another two AHE patients were residing in Nepal. Two serum samples were collected from each of the latter two patients. The four Nepalese serum samples were provided by Genelabs Diagnostics, Singapore, Singapore, and considered four independent samples.

Patients with liver diseases. Serum samples from 160 patients with liver diseases other than AHE (26 with acute hepatitis A, 27 with acute hepatitis B, 27 with acute hepatitis C, 34 with acute hepatitis D, 6 with autoimmune hepatitis, 35 with chronic hepatitis B with acute exacerbation, and 5 with primary biliary cirrhosis) were collected from persons admitted to Taipei VGH during the same period as the AHE patients. All 160 patients tested negative for HEV RNA. The diagnosis of acute hepatitis A or B was based on the presence of IgM anti-HAV or IgM anti-HBc, respectively. The diagnosis of acute hepatitis C or D was based on criteria reported previously (12, 16). The diagnosis of chronic hepatitis B with acute exacerbation was based on a history of the patient being a chronic HBV carrier, being negative for IgM anti-HBc, having a serum transaminase level higher than 10 times the upper normal limit, and having no other viral superinfections. The diagnosis of primary biliary cirrhosis or autoimmune hepatitis was based on the presence of antimitochondrial antibody or any one of the autoantibodies (antinuclear and anti-smooth muscle antibodies) and characteristic liver histological findings.

Healthy controls. Serum samples were collected from 271 healthy subjects (7 to 87 years old) who visited Taipei VGH for the hepatitis B virus vaccination program or for routine health examinations. All sera were negative for HBsAg and anti-HCV. In order to be used as a truly healthy control group for the study of the specificities of IgG and IgM anti-HEV assays in the diagnosis of AHE, these sera were further tested for HEV RNA; all were found to be negative. For the study of the rate of seroprevalence of anti-HEV in Taiwan, additional serum samples from 400 healthy subjects at two southern medical centers in Taiwan (200 subjects from National Cheng-Kung University Hospital and 200 subjects

TABLE 1. Sensitivities of IgG and IgM anti-HEV ELISA for samples from patients with AHE[a] verified by HEV RNA testing

ELISA	No. of sera that were		% Sensitivity
	Positive[b]	Negative[b]	
Anti-HEV IgG	13	2	86.7
Anti-HEV IgM	8	7	53.3

[a] Includes four serum samples from two Nepalese AHE patients.
[b] All serum samples were taken from patients with HEV RNA detectable by RT-PCR testing.

from Kaohsiung Medical University Hospital) were also included. The latter healthy control serum samples were also negative for HBsAg and anti-HCV. These sera were not tested for HEV RNA. All samples were preserved at −70°C until used.

IgG and IgM anti-HEV ELISA. All serum samples were thawed at room temperature and tested with IgG and IgM anti-HEV ELISA kits (manufactured by Genelabs Diagnostics). Fusion proteins M 3-2, B 6-1-4, and M 4-2, corresponding to the immunodominant epitopes found in ORF2 and ORF3 of the Mexico strain and the Burma strain, were used to coat the solid phase of the ELISA to detect IgG and IgM anti-HEV (17). The ELISA were performed according to the protocols provided by the manufacturer.

Detection of serum HEV RNA by RT-PCR. Serum HEV RNA was reverse transcribed to generate cDNA using random primers. The cDNA was divided for PCR using different sets of primers. Nested PCR used to detect HEV RNA with two sets of primers (F1 and R1 in the first round and F2 and R2 in the second round) was carried out as previously described (11, 32). Another two sets of primers (set 1 primers, external 3,156 and 3,157, internal 3,158 and 3,159; set 2 primers, external 3,160 and 3,161, internal 3,162 and 3,163) were also used in nested PCR as previously described (21). The sensitivity of RT-PCR for detecting HEV RNA is 10 copies. Strict procedures were followed to avoid false-positive results (15). The amplified PCR products had been cloned, sequenced, and deposited in GenBank previously (31, 32).

Serological assays. The following viral markers were tested with radioimmunoassay kits: IgM anti-HAV, HBsAg, IgM anti-HBc, and antibody to hepatitis D antigen (HAVABM, Ausria II-125, CORAB-M, and anti-Delta, respectively; Abbot Laboratories, North Chicago, Ill.). Anti-HCV was tested with a second-generation enzyme immunoassay (Abbot). Serum alanine transaminase, albumin, bilirubin (total and direct), alkaline phosphatase, and γ-glutamyltranspeptidase were measured with a sequential multiautoanalyzer (SMAC; Technicon Instruments Corporation, Tarrytown, N.Y.).

Statistical analysis. The IgG and IgM anti-HEV tests were compared with the HEV RNA test by RT-PCR for concordance. Fisher's exact test and the chi-square test were used to compare the prevalence of anti-HEV among groups. A P value of less than 0.05 was considered significant.

RESULTS

Sensitivities of IgG and IgM anti-HEV assays. The sensitivity of the IgG anti-HEV assay was determined to be 86.7% for the diagnosis of AHE, while that of the IgM anti-HEV assay was only 53.3% (Table 1). The two sera that tested negative for IgG anti-HEV were found to be positive with the same assay in our previous study (32). Of the six IgM anti-HEV-negative AHE patients residing in Taiwan (patients 6 to 11; Table 2), five had a history of traveling to endemic countries before the onset of illness. Sera were collected for anti-HEV assays more than 14 days after the occurrence of peak alanine transaminase levels for three of the six IgM anti-HEV-negative AHE patients, while such late collection was reported for only one of the five IgM anti-HEV-positive patients. As shown in Table 2, all six IgM anti-HEV-negative patients from Taiwan also had low or negative IgG anti-HEV optical densities ($P = 0.00216$, compared with the five IgM anti-HEV-positive patients). Among the four Nepalese sera, all were positive for IgG anti-HEV, but one of them was negative for IgM anti-HEV. This IgM-negative serum was obtained 17 days after the initial IgM-positive sera from the same Nepalese patient (patient 15; Table 2).

Specificities of IgG and IgM anti-HEV assays. The study of the seroprevalence of IgG and IgM anti-HEV for the 671 sera obtained from three medical centers resulted in 74 (11%) testing positive for IgG anti-HEV and 25 (3.7%) testing positive for IgM anti-HEV (Table 3). The rate of prevalence of IgG anti-HEV increased with age among healthy controls, rising from 2.3% for the group 20 years old and younger to 22.0% for the group 60 years old and older. Nonetheless, most IgM anti-HEV were detected in young subjects (Table 3). There was no significant difference in the prevalence of anti-HEV in age-matched groups from the three medical centers.

Among the 160 sera from HEV RNA-negative patients with acute or chronic liver diseases, 15 (9.4%) were positive for IgG anti-HEV and 3 (1.9%) were positive for IgM anti-HEV (Table 4). None was significantly different from those of healthy controls. Of the 15 patients who had other liver diseases and who had detectable IgG anti-HEV, 2 had been to China before the onset of illness. The remaining 13 patients denied any history of traveling to endemic countries. Three (11.5%) acute

TABLE 2. Clinical and laboratory data for HEV RNA-positive AHE patients

Patient[a]	Age (yr)/sex[b]	IgG anti-HEV		IgM anti-HEV		Travel history	Days[d]
		Reactivity	O.D.[c]	Reactivity	O.D.		
1	35/F	+	4.102	+	0.848	Mexico	5
2	38/M	+	4.141	+	1.040	Philippines	4
3	67/M	+	3.560	+	1.196	None	12
4	57/M	+	3.726	+	0.752	China	6
5	72/M	+	3.882	+	0.618	China	14
6	31/M	+	1.375	−	0.137	China	14
7	74/M	+	0.950	−	0.149	China	4
8	60/M	+	0.474	−	0.147	China	3
9	65/M	+	0.857	−	0.259	China	20
10	48/M	−	0.043	−	0.041	Indonesia	14
11	28/F	−	0.113	−	0.053	None	1
12	27/M	+	4.000	+	3.108	NA[e]	NA
13	27/M	+	4.000	+	1.615	NA	NA
14	23/M	+	4.000	+	3.645	NA	NA
15	23/M	+	4.000	−	0.234	NA	NA

[a] Samples 12 to 15 were obtained from two Nepalese AHE patients. Sample 13 was obtained later than sample 12 from the same Nepalese patient. Samples 14 and 15 came from another Nepalese patient, and sample 15 was obtained 17 days later than sample 14.
[b] F, female; M, male.
[c] O.D., optical density.
[d] Days from occurrence of peak levels of serum alanine transaminase to serum sampling.
[e] NA, not available.

TABLE 3. Seroprevalence of IgG and IgM anti-HEV in different age groups in healthy controls[a]

Age (y)	No. of patients	No. (%) of patients with seroprevalence for:	
		IgG anti-HEV[b]	IgM anti-HEV
<20	131	3 (2.3)	7 (5.3)
20–39	208	15 (7.2)	11 (5.3)
40–59	164	19 (11.6)	6 (3.7)
≥60	168	37 (22.0)	1 (0.6)
Total	671	74 (11.0)	25 (3.7)

[a] A total of 271 serum samples collected at Taipei VGH were negative for HEV RNA. Another 400 serum samples collected at the other two medical centers were directly used for a seroprevalence study without determination of HEV RNA.
[b] P value, <0.01.

TABLE 4. Seroprevalence of IgG and IgM anti-HEV in other liver disease controls[a]

Liver disease	Patients		No. (%) of patients with seroprevalence for:	
	No.	Age (yr)[b]	IgG anti-HEV	IgM anti-HEV
Acute				
Hepatitis A	26	30 ± 11	1 (3.8)	3 (11.5)
Hepatitis B	27	35 ± 15	0 (0)	0 (0)
Hepatitis C	27	49 ± 17	3 (11.1)	0 (0)
Hepatitis D	34	43 ± 18	5 (14.7)	0 (0)
Chronic				
Chronic hepatitis B with acute exacerbation	36	46 ± 16	5 (14.3)	0 (0)
Autoimmune hepatitis	6	42 ± 21	1 (16.7)	0 (0)
Primary biliary cirrhosis	5	63 ± 10	0 (0)	0 (0)

[a] All serum samples were negative for HEV RNA.
[b] Mean ± SD.

hepatitis A patients whose sera tested positive for IgM anti-HEV had histories of travel to Indonesia, Malaysia, and China within 2 weeks prior to the onset of illness.

As shown in Table 5, the specificities of IgG and IgM anti-HEV assays for diagnosing AHE were 92.1 and 98.6%, respectively.

DISCUSSION

In this study, we found a fairly good sensitivity (86.7%) of the IgG anti-HEV assay for the diagnosis of AHE verified by HEV RNA. However, the sensitivity (53.3%) of the IgM anti-HEV assay appeared to be less satisfactory. In previous reports, anti-HEV detection had a wide range of sensitivity and poor concordance among different assays (20). Most studies used positive control serum from acute non-A, non-B, and non-C hepatitis patients living in HEV outbreak countries or animal sera from HEV-inoculated nonhuman primates. Most of these sera had not been further verified by the presence of HEV RNA. In the current study, we used HEV RNA-positive human sera as positive control samples. This strict design could exclude non-A, non-B, non-C, non-D, and non-E hepatitis patients who had detectable anti-HEV induced from remote infection or other nonspecific antibodies cross-reacting with the HEV antigen.

The sensitivity of IgG anti-HEV in this study was comparable to that in previous reports (8, 9, 20); however, the sensitivity of IgM anti-HEV was relatively poor. Our study might underestimate the sensitivity of these assays, since the two IgG anti-HEV-negative serum samples had tested positive for IgG anti-HEV with the same kit in a previous study (32). These discrepant results might be due to low-titer antibodies having been destroyed by repeated freezing and thawing in the later study. There are three possibilities for the low sensitivity of IgM anti-HEV in this study. The first, delayed sampling, might account for negative IgM anti-HEV in some patients. Although both HEV viremia and serum IgM anti-HEV were short-lived in most patients (4, 14, 24), protracted viremia has been reported for as long as 1 to 4 months in some patients (5, 25). IgM anti-HEV might have declined to an undetectable level before the disappearance of HEV RNA. The presence of short-lived IgM anti-HEV in one of the two Nepalese AHE patients supports this possibility. The second possible explanation is sequence variations among different HEV genotypes. It was reported that IgM anti-HEV were not detectable in a patient infected with HEV strain US-1 using an assay based on Burmese and Mexican strains (27). It is likely that IgM anti-HEV also might have been undetectable in some of our patients infected with genotype 4 HEV using the same assay based on different genotypes (31, 32). Finally, a poor host immune response to HEV infection might also account for undetectable IgM anti-HEV in some of our AHE patients, as evidenced by lower IgG anti-HEV optical density values in AHE patients who were negative for IgM anti-HEV (Table 2).

HEV epidemics have not been reported in Taiwan. Recently, sanitation measures have improved, and the prevalence of antibodies to hepatitis A virus has markedly decreased in this area. It is interesting that anti-HEV is highly prevalent in the general population, especially among the elderly. Most HEV-infected patients in this study had a history of traveling to endemic countries (32). However, most healthy subjects who were seropositive for anti-HEV denied a history of foreign travel. In recent reports, a zoonotic native strain of HEV has been described. A swine HEV strain has recently been identified and cloned in the United States and shown to be highly homologous to the native human HEV strain (21). The swine HEV strain could infect primates experimentally and might have the capacity to cross-infect human beings (23). About 2% of young pigs were found to have HEV viremia in Taiwan, and the isolated Taiwanese swine HEV strain is also highly identical to human HEV strains in nucleotide acid and amino acid sequences (31). Anti-HEV have been found to be highly prevalent in pig handlers (22). All of these findings imply that zoonotic spreading of HEV infection between persons and pigs is possible. Recently, rats have also been found seropositive for anti-HEV and are considered to be responsible for spreading HEV among city residents in the United States (13). The high IgG anti-HEV seroprevalence rate in Taiwan might be due to remote subclinical infection during travel to endemic areas (1, 32) or zoonotic infection locally (31).

The presence of seropositive IgM anti-HEV usually indicates HEV infection. In our study, as many as 25 healthy subjects (3.7%) had detectable serum IgM anti-HEV. This result was similar to the rate of seroprevalence of IgM anti-HEV in Hong Kong (18). Those authors speculated that the seropositive results for IgM anti-HEV in asymptomatic subjects were caused by recent subclinical infection. However, we were unable to detect HEV RNA in any of the healthy controls who were positive for IgM anti-HEV. In an animal study, HEV RNA was also undetectable in naturally infected rats with seropositive IgM anti-HEV in the United States (13). IgM anti-HEV could last for 6 to 7 months in some patients after HEV infection (8). It was difficult to collect sera from asymptomatic subjects in the short viremic period after exposure to HEV. In addition, the variation of the nucleotide sequence in

TABLE 5. Specificity of IgG and IgM anti-HEV ELISA in the differential diagnosis of AHE in healthy and liver disease controls[a]

ELISA	% Specificity (S) and % concordance (C) for comparison of 15 AHE samples with the following control samples:					
	Other liver disease (n = 160)		Healthy (n = 271)		All (n = 431)	
	S	C	S	C	S	C
IgG anti-HEV	90.6	90.3	93.0	92.7	92.1	91.9
IgM anti-HEV	98.1	94.3	98.9	96.5	98.6	97.1

[a] All control serum samples were negative for HEV RNA.

the primer regions among different HEV strains could be as high as 28%, which may account for the difficulty in PCR amplification of viral sequences (11). Nevertheless, the possibility of false-positive IgM anti-HEV in these subjects could not be excluded. Further studies with a consensus primer may be needed.

Among patients with other liver diseases, IgM anti-HEV were exclusively found in three patients with anti-hepatitis A. Dual infection with acute hepatitis A and AHE has been reported previously because both share the same fecal-oral transmission route (6). Although HEV RNA was undetectable in the three acute hepatitis A patients, dual infection was possible because they had been traveling to HEV-endemic areas before the onset of illness. On the contrary, most patients who were positive for IgG anti-HEV and negative for HEV RNA had no history of travel to HEV-endemic areas. The latter group might have had remote subclinical infections because IgG anti-HEV are long-lived (24, 30). Long-lived IgG anti-HEV can persist for years and can account for the high rates of seroprevalence in older subjects among the general population (1, 24).

In conclusion, IgG anti-HEV testing has fairly good specificity and sensitivity in detecting acute HEV infection. This anti-HEV assay has good concordance with HEV RNA testing by RT-PCR. The IgG anti-HEV test can be used to screen for AHE in nonendemic areas. The IgM anti-HEV test, with its better specificity, is of some help for confirming AHE infection in IgG anti-HEV-positive patients if RT-PCR testing is not available. The high rate of prevalence of anti-HEV in healthy controls indicates that subclinical infection may exist.

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