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Simultaneous Detection of Immunoglobulin A (IgA) and IgM Antibodies against Hepatitis E Virus (HEV) Is Highly Specific for Diagnosis of Acute HEV Infection

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Serum samples collected from 68 patients (age, mean ± the standard deviation [SD], 56.3 ± 12.8 years) at admission who were subsequently molecularly diagnosed as having hepatitis E and from 2,781 individuals who were assumed not to have been recently infected with hepatitis E virus (HEV; negative controls; 52.9 ± 18.9 years), were tested for immunoglobulin M (IgM) and IgA classes of antibodies to HEV (anti-HEV) by in-house solid-phase enzyme immunoassay with recombinant open reading frame 2 protein expressed in the pupae of silkworm as the antigen probe. The 68 patients with hepatitis E had both anti-HEV IgM and anti-HEV IgA. Among the 2,781 controls, 16 (0.6%) had anti-HEV IgM alone and 4 (0.1%) had anti-HEV IgA alone: these IgA/IgM anti-HEV-positive individuals were not only negative for HEV RNA but lack IgG anti-HEV antibody as well (at least in most of the cases). Periodic serum samples obtained from 15 patients with hepatitis E were tested for HEV RNA, anti-HEV IgM, and anti-HEV IgA. Although HEV RNA was detectable in the serum until 7 to 40 (21.4 ± 9.7) days after disease onset, both IgM and IgA anti-HEV antibodies were detectable until 37, 55, or 62 days after disease onset in three patients and up through the end of the observation period (50 to 144 days) in 12 patients. These results indicate that detection of anti-HEV IgA alone or along with anti-HEV IgM is useful for serological diagnosis of hepatitis E with increased specificity and longer duration of positivity than that by RNA detection.

Hepatitis E, the major form of enterically transmitted non-A, non-B hepatitis, is caused by hepatitis E virus (HEV). HEV is transmitted primarily by the fecal-oral route. Waterborne epidemics are characteristic of hepatitis E in developing regions of Africa, the Middle East, and Southeast and Central Asia, where sanitation conditions are suboptimal; one epidemic has also been documented in North America (Mexico) (32). HEV-associated hepatitis also occurs among individuals in industrialized countries with no history of travel to areas where HEV is endemic (6, 9, 18, 25, 36, 37, 39, 41, 52, 54). Recently, accumulating lines of evidence indicate that hepatitis E is a zoonosis, and pigs or other animals may act as reservoirs for HEV infection in humans (9, 15, 20–24, 27, 39, 42, 45, 56). A significant proportion of healthy individuals in industrialized countries where hepatitis E is not endemic are seropositive for HEV antibodies (8, 19, 46). Therefore, several epidemiological questions remain unanswered. The success of future studies on clinical and subclinical HEV infection not only in developing countries but also in industrialized countries will greatly depend on the availability of assays that are sensitive and specific.

HEV was recently classified as the sole member of the genus *Hepevirus* in the family *Hepeviridae*. The genome of HEV is a 7.2-kb, positive-sense, single-stranded RNA. It contains a short 5′ untranslated region, three open reading frames (ORFs; ORF1, ORF2 and ORF3), and a short 3′ untranslated region terminated by a poly(A) tract (12, 34, 44, 53). ORF1 encodes nonstructural proteins, ORF2 encodes the capsid protein, and ORF3 encodes a cytoskeleton-associated phosphoprotein. Extensive diversity has been noted among HEV isolates, and HEV sequences have been classified into four major genotypes (genotypes 1 to 4) (37). In Japan, polyphyletic HEV strains of genotype 3 or 4 or both have been isolated from patients with sporadic acute or fulminant hepatitis E who had no history of travel to countries where this virus is endemic (1, 25, 30, 40, 41, 56).

The immunoglobulin M (IgM) class of antibody against HEV (anti-HEV IgM) is used as a reliable and sensitive marker of recent HEV infection (2–4, 38). However, the specificity of the solid-phase assay for anti-HEV IgM has been questioned in some cases, particularly in patients with IgM-rheumatoid factors in the serum, which have activity against the Fc portion of IgG directed to HEV antigen and may elicit

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a false-positive result (10). Virus-specific IgA class antibodies have been detected during the acute stage of infection with hepatitis A virus (HAV) (57) or hepatitis B virus (HBV) (28). The IgA class of antibodies has also been detected in sera from patients with hepatitis E (2, 14, 47). Although a few previous studies reported that anti-HEV IgA could be utilized as an additional confirmatory antibody for recent HEV infection (2, 47), the clinical and epidemiological implications of positivity for anti-HEV IgA remain to be clarified.

Therefore, in the present study, we compared the sensitivity and specificity of the anti-HEV IgA and anti-HEV IgM assays and evaluated their ability to diagnose hepatitis E by using serum samples from 68 patients who were subsequently molecularly diagnosed as having hepatitis E and from 2,781 individuals who were assumed to not have been recently infected with HEV as negative controls in an attempt to improve the serological diagnosis of recent HEV infection that is occurring epidemically in developing countries and more frequently than previously thought in industrialized countries, including Japan.

MATERIALS AND METHODS

Serum samples. The present study included serum samples obtained from 68 patients (56 males and 12 females; age, mean ± the standard deviation [SD], 56.3 ± 12.8 [range, 25 to 86] years) at admission who had detectable HEV RNA and who were subsequently diagnosed as having sporadic acute or fulminant hepatitis E. Thirty of the 68 patients had been included in our previous studies for detection of HEV RNA and phylogenetic analysis of HEV isolates (1, 17, 25, 40, 41, 55, 56). The present study also included periodic serum samples collected from 15 of the HEV-infected patients, from whom one or more serum samples had been obtained during each of the following periods: between 0 and 10 days, between 20 and 40 days, and between 50 and 70 days after the onset of the illness. In addition, serum samples obtained from 2,781 individuals who were assumed to not have been recently infected with HEV (1,282 males and 1,499 females; 52.9 ± 18.9 [0 to 97] years) were used as negative controls, and they included 675 samples from voluntary blood donors with normal alanine aminotransferase level (328 males and 347 females; 39.0 ± 16.0 [16 to 64] years), 127 samples from patients with type A, type B, or type C acute hepatitis (77 males and 50 females; 35.7 ± 12.4 [16 to 78] years), 274 samples from patients with type B or type C chronic liver disease (158 males and 116 females; 55.0 ± 13.6 [23 to 83] years), 472 samples from patients on maintenance hemodialysis (262 males and 210 females; 59.0 ± 12.4 [24 to 94] years), 147 samples from patients with primary biliary cirrhosis (27 males and 120 females; 59.7 ± 10.7 [31 to 79] years), 186 samples from patients with rheumatoid arthritis (21 males and 165 females; 63.8 ± 13.2 [26 to 91] years), and 900 samples from patients (409 males and 491 females; 58.5 ± 20.7 [0 to 97] years) who received a routine health examination or care for various disorders at one of our hospitals.

The presence of IgM class antibodies to HAV (anti-HAV IgM), antibodies to HBV core IgM, hepatitis B surface antigen (HBsAg), and antibodies to hepatitis C virus (HCV) (anti-HCV) was determined by commercially available kits (HAVAB-M and CORZYME-M [Abbott Laboratories, Abbott Park, Ill.], Mycell II HBsAg [Institute of Immunology Co., Ltd., Tokyo, Japan], and Abbott HCV PHA Second Generation [Abbott Japan, Tokyo, Japan]). The presence of HBV DNA and HCV RNA was determined by the methods described previously (29, 43). The study protocol conformed to the ethical guidelines and was approved by the ethics committees of the institutions. Informed consent was obtained from each patient.

ELISAs for detecting anti-HEV antibodies. Our previously described in-house enzyme-linked immunosorbent assays (ELISAs) methods for detection of IgM and IgA anti-HEV antibodies using purified recombinant ORF2 protein (25) were performed with the following modifications. Wells of microplates (part no. 762071; Greiner Bio-One GmbH, Frickenhausen, Germany) were coated with 50 μl of the recombinant ORF2 protein (5 μg/ml in phosphate-buffered saline [pH 7.5]) and incubated at room temperature overnight. After removal of the coating buffer, 100 μl of 10 mM Tris-buffered saline (pH 7.5) containing 2.5% (vol/vol) Block Ace (Dainippon Pharmaceutical Co., Ltd., Osaka, Japan) and 0.18% Tween 20 was added. The microplates were incubated at room temperature for 4 h. The blocking buffer was discarded, and each well was washed five times with saline containing 2% lactose (Kanto Chemical Co., Inc., Tokyo, Japan) and then freeze-dried. To test for anti-HEV IgM, 50 μl of each sample was added to each well at a dilution of 1:100 in 10 mM Tris-buffered saline containing 40% Block Ace, 0.18% Tween 20, and a mock protein (optical density [OD] at 280 nm = 0.1) that had been obtained from the pupae of silkworm infected with nonrecombinant baculovirus. The microplates were incubated at room temperature for 1 h and were then washed five times with washing buffer (saline with 0.05% Tween 20). A total of 50 μl of phosphate-buffered saline containing 25% (vol/vol) fetal bovine serum (Sigma Chemical, St. Louis, Mo.) and peroxidase-conjugated mouse monoclonal anti-human IgM (M-49; Institute of Immunology Co., Ltd.) (50) was added to each well. The microplates were incubated at room temperature for 1 h and then washed five times with washing buffer. Then, 50 μl of tetramethylbenzidine-soluble reagent (BioFX Laboratories, Inc., Owings Mills, Md.) as a substrate was added to each well. The plate was incubated at room temperature for 30 min in the dark, and then 50 μl of tetramethylbenzidine stop buffer (BioFX Laboratories, Inc.) was added to each well. The OD value of each sample was read at 450 nm. For the anti-HEV IgA assay, peroxidase-labeled mouse monoclonal anti-human IgA (A-13; Institute of Immunology Co., Ltd.) (28) was used in place of the enzyme-labeled anti-human IgM. Test samples with OD values equal to or greater than the cutoff value were considered to be positive for anti-HEV IgM or anti-HEV IgA.

In addition, anti-HEV IgG was assayed according to the method described previously, and the cutoff value used for the anti-HEV IgG assay was 0.152 (25).

The specificity of the anti-HEV assays was verified by absorption with the same recombinant ORF2 protein (50 μg/ml at the final concentration for anti-HEV IgG or anti-HEV IgA assay; 150 μg/ml at the final concentration for anti-HEV IgM assay) that was used as the antigen probe. Briefly, prior to testing, the serum sample was diluted 1:100, 1:300, 1:1,000, 1:3,000, 1:10,000, or 1:30,000 to adjust its OD value to <1.5. If the OD value of the tested sample was reduced by ≥50% in the anti-HEV IgM assay or ≥70% in the anti-HEV IgA or IgG assay after absorption with the recombinant ORF2 protein, the sample was considered to be positive for anti-HEV.

Detection of HEV RNA. Reverse transcription-PCR (RT-PCR) was performed for detection of HEV RNA in serum. Total RNA was extracted from 100 μl of serum, reverse transcribed, and then subjected to nested PCR with ORF2 primers as described previously (25, 42). The size of the amplification product of the first-round PCR was 506 bp and that of the second-round PCR was 457 bp. The nested RT-PCR assay used had the capability of amplifying all four known genotypes of HEV strains reported thus far (25, 42, 56). The RT-PCR assay was performed in duplicate, and the reproducibility was confirmed. The specificity and sensitivity of the RT-PCR assay were assessed as described previously (25, 42).

RESULTS

Determination of the cutoff values for the anti-HEV IgM and anti-HEV IgA assays. Since the prevalence of HEV infection in the southern part of Japan is low (8, 30), it was assumed that voluntary blood donors in Yamaguchi Prefecture, which is located in the southern part of mainland Honshu of Japan, were highly unlikely to have been infected with HEV in the period just prior to their donating blood. Therefore, to determine the cutoff values for the anti-HEV IgM and anti-HEV IgA assays, serum samples from 675 donors with a normal alanine aminotransferase level who donated blood at the Japanese Red Cross Blood Center in Yamaguchi Prefecture were used as a panel in the present study. In the anti-HEV IgM assay, the OD values ranged from 0.001 to 0.542, and the value of 0.440, which was calculated as 7 SD above the mean value (0.072), was used as the tentative cutoff value. In the anti-HEV IgA assay, OD values ranging from 0.000 to 1.754 were obtained from the 675 control sera; the OD value of 0.642 (mean + 7 SD) was used as the cutoff value for anti-HEV IgA.

Although 16 (2.4%) of the 675 serum samples were positive for anti-HEV IgG (Table 1), the 16 samples tested negative for HEV RNA, and their OD values ranged from 0.036 to 0.161 (below the cutoff value) in the anti-HEV IgM assay and from 0.019 to 0.180 (below the cutoff value) in the anti-HEV IgA

TABLE 1. Prevalence of anti-HEV IgM and anti-HEV IgA among various groups of subjects

Group	No. of subjects studied	Age (yr) (mean ± SD)	No. (%) of subjects with:			
			Anti-HEV IgG[a]	Anti-HEV IgM	Anti-HEV IgA	Both anti-HEV IgM and anti-HEV IgA
Blood donors with normal ALT	675	39.0 ± 16.0	16 (2.4)	1 (0.1)	1 (0.1)	0
Patients with acute hepatitis	127	35.7 ± 12.4	11 (8.7)	4 (3.1)	2 (1.6)	0
Type A	57	35.1 ± 9.3	7 (12.3)	4 (7.0)	1 (1.8)	0
Type B	61	34.6 ± 12.9	3 (4.9)	0	1 (1.6)	0
Type C	9	47.6 ± 20.4	1 (11.1)	0	0	0
Patients with chronic liver diseases	274	55.0 ± 13.6	26 (9.5)	2 (0.7)	0	0
Chronic hepatitis	182	51.2 ± 13.4	15 (8.2)	1 (0.5)	0	0
Liver cirrhosis	57	62.9 ± 10.2	7 (12.3)	1 (1.8)	0	0
Hepatocellular carcinoma	35	61.7 ± 11.2	4 (11.4)	0	0	0
Hemodialysis patients	472	59.0 ± 12.4	60 (12.7)	2 (0.4)	0	0
Patients with primary biliary cirrhosis	147	59.7 ± 10.7	15 (10.2)	4 (2.7)	0	0
Patients with rheumatoid arthritis	186	63.8 ± 13.2	6 (3.2)	3 (1.6)	0	0
Hospital patients[b]	900	58.5 ± 20.7	24 (2.7)	0	1 (0.1)	0
Total of control subjects[c]	2,781	52.9 ± 18.9	158 (5.7)	16 (0.6)	4 (0.1)	0
Patients with hepatitis E	68	56.3 ± 12.8	68 (100)	68 (100)	68 (100)	68 (100)

[a] Positivity for anti-HEV IgG was confirmed in all 226 samples by the absorption test (see Materials and Methods).
[b] They received a routine health examination or care for various disorders at one of our hospitals.
[c] They were assumed not to have been recently infected with HEV.

assay, suggesting the absence of present HEV infection in the studied population.

Detection of anti-HEV IgM and anti-HEV IgA in individuals who were assumed not to have been infected recently with HEV. Among the serum samples obtained from the above-mentioned 675 donors, only one sample was positive for anti-HEV IgM with an OD value of 0.542, and a different sample was positive for anti-HEV IgA alone with an OD value of 1.754 (Table 2). However, these two serum samples were negative for anti-HEV IgG and HEV RNA, suggesting that the anti-HEV IgM or IgA was falsely detected in these two samples.

Using the cutoff values described above, the remaining 2,106 serum samples obtained from 127 patients with type A, type B, or type C acute hepatitis, 274 patients with type B or type C chronic liver disease, 472 patients on maintenance hemodialysis, 147 patients with primary biliary cirrhosis, 186 patients with rheumatoid arthritis, and 900 patients who received routine health examination or medical care for various disorders

TABLE 2. Serum samples that were falsely positive for anti-HEV IgM or IgA in the in-house ELISAs used in the present study

Sample ID no.	Diagnosis	Age (yr)/sex[a]	OD at 450 nm[b]			HEV RNA
			Anti-HEV IgM	Anti-HEV IgA	Anti-HEV IgG	
389	Blood donor	59/M	**0.542 (8)**	0.038	0.075	–[c]
674	Blood donor	55/F	0.024	**1.754 (5)**	0.025	–
868	Health check-up	62/M	0.078	**0.946 (9)**	0.034	–
1614	Hemodialysis	55/F	**2.541 (8)**	0.056	0.089	–
1761	Hemodialysis	84/M	**1.018 (23)**	0.194	0.259 (83) (+)	–
2110	Acute hepatitis (type A)	21/F	**1.986 (−7)**	0.173	1.824 (92) (+)	–
2136	Acute hepatitis (type A)	28/F	**1.509 (−6)**	0.042	0.036	–
2113	Acute hepatitis (type A)	43/F	**0.559 (25)**	0.046	0.089	–
2102	Acute hepatitis (type A)	31/F	**0.445 (5)**	0.079	**0.388 (−1)**	–
2099	Acute hepatitis (type A)	34/M	0.286	**0.731 (6)**	0.037	–
2061	Acute hepatitis (type B)	55/M	0.055	**0.692 (−6)**	0.028	–
2201	Primary biliary cirrhosis	48/F	**1.215 (20)**	0.068	0.049	–
2229	Primary biliary cirrhosis	61/F	**0.545 (−6)**	0.072	0.054	–
2232	Primary biliary cirrhosis	60/F	**0.470 (9)**	0.083	0.068	–
2257	Primary biliary cirrhosis	52/F	**0.462 (17)**	0.081	0.023	–
2463	Rheumatoid arthritis	70/M	**0.933 (8)**	0.106	0.071	–
2496	Rheumatoid arthritis	83/F	**0.785 (22)**	0.038	0.022	–
2545	Rheumatoid arthritis	71/F	**0.522 (18)**	0.103	0.024	–
2919	Liver cirrhosis (type C)	61/M	**0.594 (28)**	0.325	0.265 (86) (+)	–
3007	Chronic hepatitis (type B)	44/M	**0.488 (2)**	0.020	0.008	–

[a] M, male; F, female.
[b] If the OD value of the tested sample was reduced by only <50% in the anti-HEV IgM assay or < 70% in the anti-HEV IgA or IgG assay after absorption with the recombinant ORF2 protein, the result was considered to be false positive, and such samples are indicated in boldface. Numbers in parentheses are percent values.
[c] —, no HEV RNA was detected.

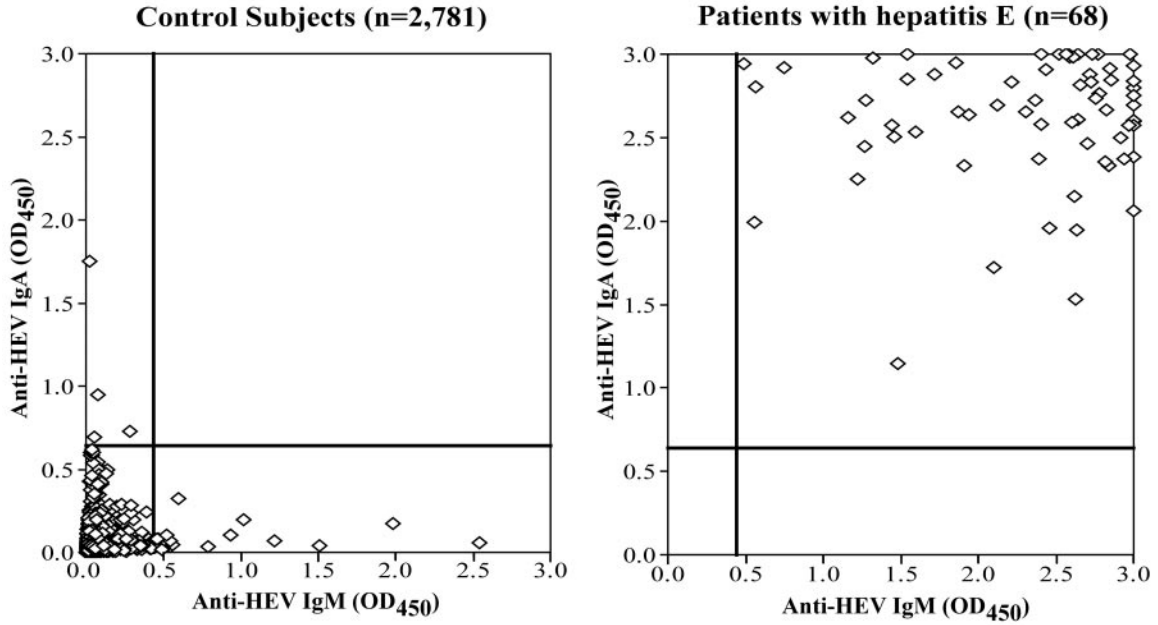

FIG. 1. Distribution of OD values from two ELISAs for anti-HEV IgM and anti-HEV IgA among patients with hepatitis E and among the control subjects. Serum samples from 2,781 subjects who were assumed not to have been infected recently with HEV and from 68 patients with hepatitis E were concurrently tested by the solid-phase ELISAs for anti-HEV IgM and anti-HEV IgA. Horizontal and vertical lines represent the cutoff values for anti-HEV IgA and anti-HEV IgM, respectively.

at one of our hospitals, were tested for anti-HEV IgM and anti-HEV IgA (Table 1). Among the 2,781 subjects who were assumed not to have been infected recently with HEV, including the 675 blood donors described above, anti-HEV IgM was detected in the serum samples from 16 subjects (0.6% or 16 of 2,781), including 4 patients with acute hepatitis A (7.0% or 4 of 57). Anti-HEV IgA was detected in the serum samples from four other patients (0.1% or 4 of 2,781) (Table 1), the difference being statistically significant ($P = 0.0139$ [χ^2-test]). Although the 16 samples had OD values of anti-HEV IgM greater than the cutoff value, with the OD value ranging from 0.445 to 2.541, and the other four samples had OD values of anti-HEV IgA greater than the cutoff value, with the OD value ranging from 0.692 to 1.754, positivity for HEV antibodies could not be confirmed by the absorption test in any of the 20 samples (Table 2). Furthermore, none of these 20 serum samples with anti-HEV IgM or anti-HEV IgA alone had detectable HEV RNA, indicating that these serum samples were falsely positive for anti-HEV IgM or anti-HEV IgA in the ELISAs used.

Of note, among the 2,781 samples from subjects who were assumed not to have been recently infected with HEV in the present study, no serum sample was positive for both IgM and IgA anti-HEV antibodies (Fig. 1).

Detection of anti-HEV IgM and anti-HEV IgA in patients with hepatitis E. Serum samples obtained from 68 patients with sporadic acute or fulminant hepatitis E were tested for the presence of IgM and IgA anti-HEV antibodies. All 68 patients had anti-HEV IgM with OD values ranging from 0.486 to >3.0 and anti-HEV IgA with OD values ranging from 1.146 to >3.0 (Fig. 1). The presence of anti-HEV IgM and anti-HEV IgA was confirmed by the absorption test in the serum samples from all 68 patients, indicating that patients with virologic evidence of the early phase of HEV infection are positive for both anti-HEV IgM and anti-HEV IgA, in sharp contrast to the 20 patients in the control group who had anti-HEV IgM or IgA alone. This finding suggests that the combinatorial detection of both classes of antibodies (IgM and IgA) is efficient for serological diagnosis of hepatitis E with increased accuracy. Among the 68 patients with hepatitis E, four patients (5.9%) had an OD value of <1.000 in the anti-HEV IgM assay, and only one patient had an OD value of <1.500 in the anti-HEV IgA assay. All 68 patients had high levels (1.235 to >3.000) of anti-HEV IgG, 59 (86.8%), of whom had an OD value of >2.000.

Detection of anti-HEV IgM, anti-HEV IgA, and HEV RNA in follow-up serum samples from infected patients. Figure 2 shows the HEV RNA, anti-HEV IgM, and anti-HEV IgA profiles associated with the HEV infection in 15 patients (patients 1 to 15). From these 15 patients, in addition to the serum sample obtained at admission, 3 to 30 other serum samples, including those obtained between 20 and 40 days and between 50 and 70 days after the disease onset, were available. HEV RNA remained detectable in the serum until 7 to 40 (21.4 ± 9.7) days but disappeared 15 to 59 (32.7 ± 13.4) days after the onset of the disease. Anti-HEV IgM and IgA antibodies were both detectable up through the end of the observation period (50 to 144 [72.8 ± 28.5] days after disease onset) in 12 of the 15 patients. In the remaining three patients (patients 1, 3, and 5), both IgM and IgA anti-HEV antibodies were detectable until 37, 55, and 62 days, respectively, after disease onset, but either the IgM or IgA class of anti-HEV antibodies disappeared at 44, 62, and 104 days, respectively, after the disease onset. The presence of anti-HEV IgM and anti-HEV IgA was

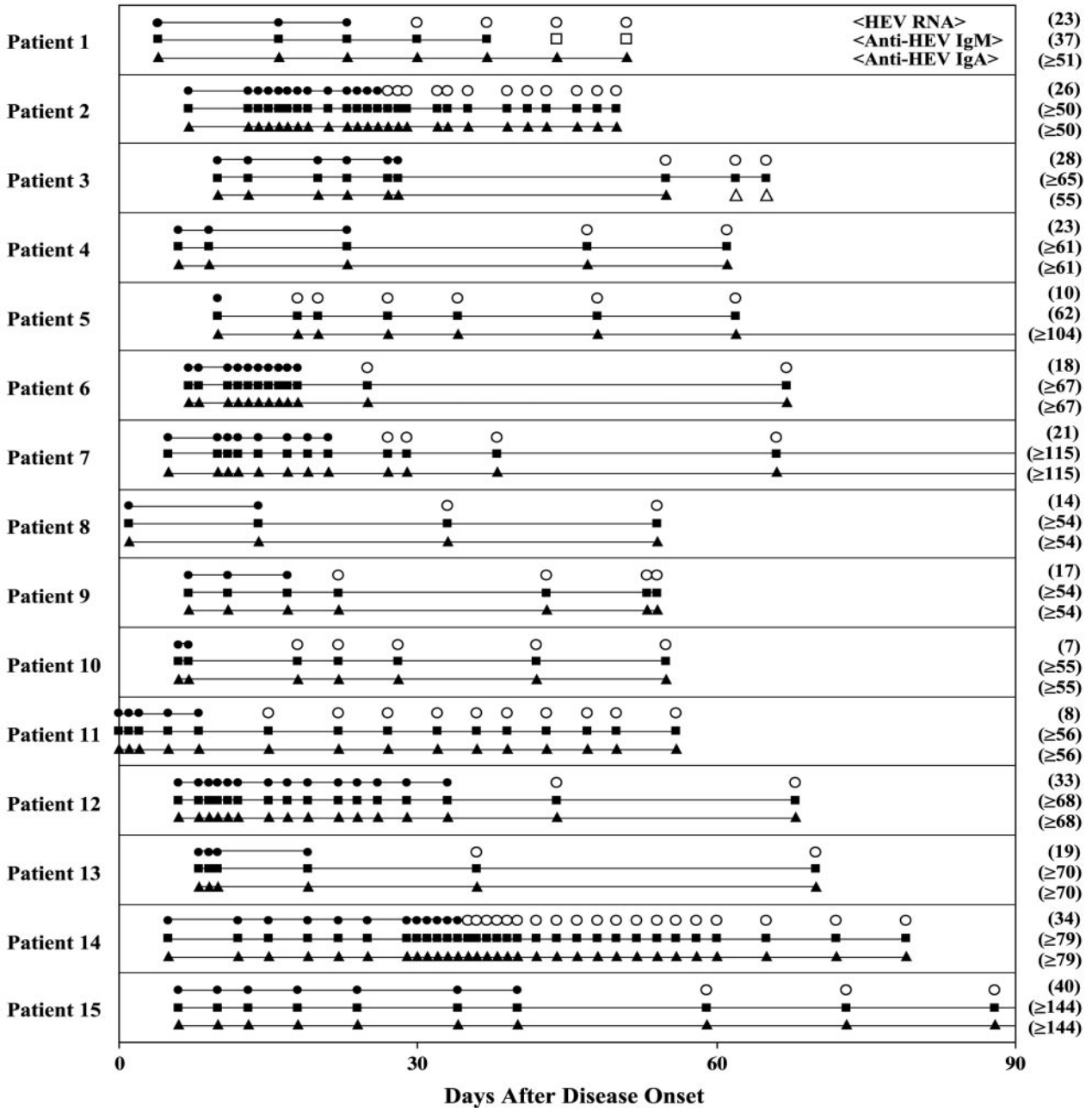

FIG. 2. Detection of HEV RNA, anti-HEV IgM and anti-HEV IgA in initial and follow-up serum samples from 15 patients (patients 1 to 15) with hepatitis E. For each patient, closed and open circles in the top row represent positivity or negativity for HEV RNA, respectively; closed and open boxes in the middle row represent positivity or negativity for anti-HEV IgM, respectively; and closed and open triangles in the bottom row represent positivity or negativity for anti-HEV IgA, respectively. The number in parentheses at the end of each row indicates the final day on which HEV RNA, anti-HEV IgM, or anti-HEV IgA was detectable. Patients 1 and 9 contracted fulminant hepatitis E and died 56 and 54 days, respectively, after onset of the illness.

also confirmed by the absorption test in the follow-up serum samples from all 15 patients, including the last two specimens from patients 1 and 3, who became positive for only anti-HEV IgA and IgM, respectively. The IgG antibody level was as high as 3.0 OD at admission in all 15 patients and persisted at a high level. There was no discernible reduction in the IgG antibody level up through the end of the observation period.

DISCUSSION

The diagnosis of acute or fulminant hepatitis E is based on detection of the HEV genome in serum or feces by RT-PCR (3, 5, 13, 25) or detection of newly elicited antibodies to HEV (3, 4, 14, 19, 25, 38, 49, 59). The presence of a specific antibody of the IgM class is diagnostic of recent or ongoing infection. As

is our in-house ELISA, a solid-phase (sandwich or indirect) ELISA method for detecting anti-HEV IgM is simple and is currently used in the majority of reported in-house ELISAs (3, 4, 14, 19, 25, 38, 49, 59), as well as in a commercial kit marketed in Asia by Genelabs Diagnostics (Singapore). One of the weaknesses of the solid-phase ELISA format is reduced sensitivity due to competition among virus-specific IgM, IgA, and IgG for antigen-binding sites. It has been pointed out that sensitivity is compromised when corresponding IgG titers are disproportionately higher than those of the IgM antibodies (16). Another potential weakness of the solid-phase test for IgM antibody is that IgM-rheumatoid factor in sera from patients with rheumatoid arthritis may elicit a false-positive result (10). Recently, to overcome these weaknesses in the solid-phase ELISAs, an IgM class capture system was introduced by Yu et al. (58). In the class capture system, competing IgG antibodies (also IgA antibodies) in the sample are eliminated at the beginning of the assay, thus enhancing the reaction between anti-HEV IgM and the HEV antigen, although its efficiency depends on the capacity of solidified antibodies against total human IgM molecules containing anti-HEV IgM to capture the HEV antigen. The class capture assay developed by Yu et al. (58) provided a reliable method for detecting anti-HEV IgM and had specificities comparable to those determined by the solid-phase assay when acute-phase sera with high anti-HEV IgM levels were tested and had higher sensitivity for samples with a low anti-HEV IgM concentration or with a high anti-HEV IgG concentration. However, as described by Seriwatana et al. (38), we had to stop developing an IgM class capture ELISA after initial experiments demonstrated poor sensitivity despite the use of substantially greater amounts of the recombinant HEV antigen and several monoclonal antibodies raised against recombinant HEV antigen with distinct specificities to detect the recombinant HEV antigen captured by anti-HEV IgM (unpublished observations). Therefore, in the present study, we chose the solid-phase ELISA format for detecting anti-HEV antibodies.

It has been reported that anti-HEV IgA can be utilized as an additional confirmatory antibody for recent HEV infection (2). Although the presence of a specific antibody of the IgA class is diagnostic for recent infection in several viral or nonviral diseases, including type A, type B, or type C acute hepatitis (28, 35, 57), as well as *Chlamydia trachomatis* infection, *Chlamydia pneumoniae* infection, and cholera (26, 33, 51), the clinical and epidemiological significance of positivity for anti-HEV IgA remains to be fully verified. In the present study, we used the IgM and IgA anti-HEV tests together to characterize serum specimens from 68 patients with acute or fulminant hepatitis E and from 2,781 subjects who were assumed to not have been recently infected with HEV as negative controls. With this dual testing, we obtained the following results. (i) Both anti-HEV IgM and anti-HEV IgA were detectable in serum samples obtained at admission from all 68 patients tested who were subsequently diagnosed molecularly as having hepatitis E (estimated sensitivity rate of the assay: 100% and 100%, respectively). (ii) Among the 2,781 serum samples collected from subjects who were assumed to not have been recently infected with HEV as negative controls, 16 samples (0.6%) were falsely positive for anti-HEV IgM alone and four samples (0.1%) were falsely positive for anti-HEV IgA alone, indicating that the false-positive rate was significantly lower in the anti-HEV IgA assay than in the anti-HEV IgM assay used ($P = 0.0139$) (the estimated specificity rates of the assays were 99.4 and 99.9%, respectively). (iii) Of the 2,781 serum samples collected from the subjects who were assumed to not have been recently infected with HEV, none was positive for both anti-HEV IgM and anti-HEV IgA (estimated specificity rate of the dual assay: 100%), indicating that an erroneous diagnosis of hepatitis E based on serological assay can be minimized by performing the anti-HEV IgM assay on samples that show positive results by the anti-HEV IgA assay or by performing combinatorial assay for anti-HEV IgA and anti-HEV IgM.

Regarding the duration of seropositivity for anti-HEV IgM, it has been reported that sera collected from patients during various hepatitis E outbreaks 3 to 4 months and 6 to 12 months after the onset of jaundice, 50 and 40%, respectively, were positive for anti-HEV IgM (7). In three cases of imported hepatitis E in Japan, the duration of seropositivity for anti-HEV IgM was 66, 112, and 154 days, respectively, from disease onset (19). Little is known about the duration of seropositivity for anti-HEV IgA in HEV-infected patients. Although the duration of observation was limited in the present study, anti-HEV IgA was detectable up through the end of the observation period (50 to 144 days after disease onset) in 14 of the 15 patients with hepatitis E and until 55 days after disease onset in the remaining one patient, suggesting that the durations of seropositivity for anti-HEV IgA and anti-HEV IgM, as determined by the assays that were used, are similar (Fig. 2).

In the circulation, IgA occurs in both monomeric and polymeric forms. Antibodies of the IgA class are unique in that they are produced in response to antigenic stimuli applied locally (48) and have distinct molecular forms. As for anti-HEV IgA, it is unclear whether our assay is detecting both dimeric secretory IgA and monomeric IgA, since the monoclonal antibody to IgA (A-13) that is used as an enzyme-labeled antibody in the present study can bind to various IgA species, such as secretory IgA and two subclasses of IgA (IgA1 and IgA2) (11, 28). However, it seems likely that only polymeric IgA antibody of either the IgA1 or the IgA2 subclass against HEV can be detected as described for IgA antibodies to hepatitis B core in type B acute hepatitis (11). Although an individual may have IgA deficiency, which may elicit a false-negative result in the anti-HEV IgA test, it has been reported that absence or deficiency ($<$1/100 of the average of normal controls) of total IgA was observed in only 4 (0.004%) of 93,020 apparently healthy blood donors and in 1 (0.01%) of 6,800 hospital patients in Japan: the absence of IgA was found at a frequency of 0.001% (31), indicating that false-negative results in the anti-HEV IgA assay due to the absence or deficiency of IgA in the circulation may be negligible.

Based on the results obtained in the present study, we conclude that, in solid-phase ELISA, the anti-HEV IgA assay is significantly more specific than the anti-HEV IgM assay with regard to ability to diagnose hepatitis E; that anti-HEV IgA could be the first-choice marker as a diagnostic indicator of recent HEV infection when the solid-phase ELISA method is used; and that the diagnostic accuracy increases when positive

results obtained by the anti-HEV IgA assay are confirmed by additional or simultaneous detection of anti-HEV IgM. However, due to the limited number of patients with hepatitis E enrolled in the present study, further studies are needed to verify our conclusions in larger cohorts.

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