IV. 研究成果の刊行物・別刷

（前掲一覧表のうちの一部を収載）

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Estimation of the mutation rate of hepatitis E virus based on a set of closely related 7.5-year-apart isolates from Sapporo, Japan

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Abstract

Since hepatitis E virus (HEV) does not persist in infected hosts or in cultured cell lines, it has been difficult to know its spontaneous mutation rate. Recently, we identified an HEV isolate in stored serum from a patient having developed hepatitis E in 1995 (JSM-Sap95), nucleotide sequence of which showed a strong resemblance to those obtained from three patients who contracted hepatitis E in 2000 and 2002 (JKK-Sap00, JYW-Sap02, and JTS-Sap02). The remarkable nucleotide similarity together with the fact that all these patients were residents of the same city, Sapporo, prompted us to hypothesize that JKK-Sap00, JYW-Sap02 and JTS-Sap02 are descendants of JSM-Sap95. Then, the mutation rate of HEV was calculated to be 1.72, 1.41, or 1.40×10^{-3} base substitutions per site per year, from JSM-Sap95 to JKK-Sap00, JYW-Sap02 or JTS-Sap02, respectively. Interestingly, these values were very similar to those (1.44×10^{-3} to 1.92×10^{-3}) reported for hepatitis C virus. Because it remains possible that JSM-Sap95 is not the direct ancestor of the other three isolates but was merely a relative of the true ancestor, HEV mutation rate may be a little lower than 1.40×10^{-3} base substitutions per site per year.
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Keywords: Mutation rate; Hepatitis E virus; HEV RNA genome; Nucleotide substitutions per site per year

1. Introduction

During these years after the discovery of a Japan-indigenous hepatitis E virus strain (HEV-JRA1) in 2001 [1], it has been revealed that the HEV strains existing in Japan are polyphyletic [2,3] and that zoonotic food-borne mode of transmission seems to play an important role [4–6]. It remains obscure, however, when and wherefrom the ancestral HEV strains made inroad in Japan. To know it, we can use the technique of molecular clock analyses. Unfortunately, however, we have not known the mutation rate of HEV, on the basis of which the molecular clock analyses could be performed.

Recently we identified an HEV strain from a case of 1995, and found that its sequence was highly homologous to those from three cases in 2000 and 2002. We hypothesized that the HEV in 1995 might be the ancestor of the three later isolates to estimate so-far unknown mutation rate of HEV.

2. Materials and methods

Sera from patients, obtained under informed consents, were kept frozen until laboratory use. The nucleotide sequence of about 7.2 kb in length encompassing complete open reading frame 1, 2, and 3 (ORF1, ORF2, and ORF3) was determined by the methods described previously [1] from three patients as follows: JSM-Sap95, 34-year-old male, whose serum was obtained 28 March 1995; JYW-Sap02, 35-year-old male, 30 August 2002; and JTS-Sap02, 41-year-old male, 14 September 2002. In addition to these, a previously sequenced isolate JKK-Sap00 from 57-year-old male at 10 November 2000 (accession number AB074917) [7] was also included for comparison. All of these patients were living in Sapporo, Hokkaido, Japan, when they contracted hepatitis E. Infection sources

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or transmission routes were totally unknown in all of the four patients.

3. Results

All of the four isolates of HEV analyzed in this study shared identical lengths of ORF1, ORF2, and ORF3, encoding 1708 amino acids (aa), 675 aa, and 115 aa, respectively. Genetic similarity among them reached higher than 99% at both nucleotide and amino acid levels, and phylogenetic tree analysis indicated that they form a very compact cluster with a bootstrap score at 100% (by 1000-time execution) within genotype IV.

Number of base substitutions between these isolates by two-by-two comparison is shown in Table 1. Obviously, the longer the time interval, the greater the difference. Alignment of the four sequences indicated that the substitutions of nucleotides observed among them were mostly "transitional" type (i.e., C to/from T, or G to/from A), and resided at common positions as shown in Figs. 1 and 2.

On the hypothesis that JKK-Sap00 in 2000 might be a mutation product of JSM-Sap95 in 1995, the mutation rate of HEV was calculated to be 1.72×10^{-3} base substitutions per site per year. Similarly, those from JSM-Sap95 to JYW-Sap02 and to JTS-Sap02 were 1.41×10^{-3} and 1.40×10^{-3} base substitutions per site per year, respectively (Fig. 3), an undistinguishable value from those reported for

Table 1
Number of nucleotide substitutions among the four isolates

Isolate	Date	Nucleotide substitutions (per elapsed days)[a]		
		JSM-Sap95	JKK-Sap00	JYW-Sap02
JSM-Sap95	28 Mar 1995			
JKK-Sap00	10 Nov 2000	69 nt (2054)		
JYW-Sap02	30 Aug 2002	75 nt (2712)	40 nt (658)	
JTS-Sap02	14 Sep 2002	75 nt (2727)	40 nt (673)	4 nt (15)

[a] Mismatched nucleotides were counted over the 7145-nt sequence which encompasses entire coding region (ORF1 + ORF2 + ORF3).

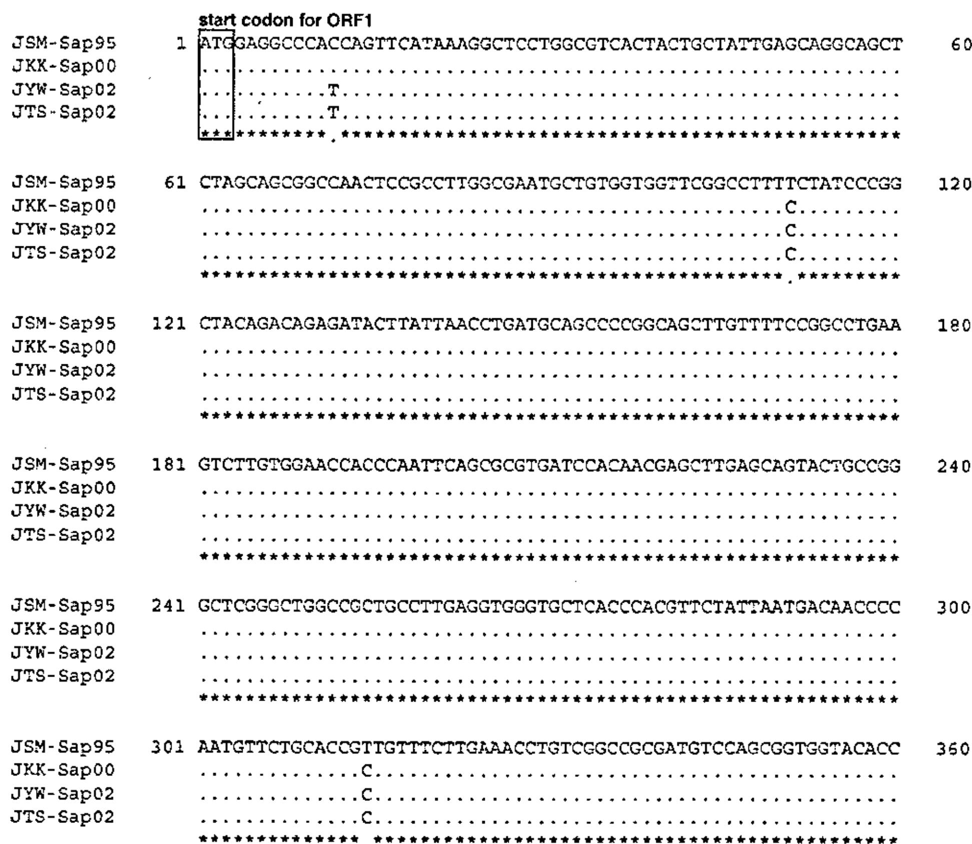

Fig. 1. Alignment of nucleotide sequences of the four isolates. Only the 5′-end 360-nt sequence of ORF1 is shown here. Entire coding region sequences can be retrieved from the DDBJ/EMBL/GenBank databases under the accession numbers AB161717 (JSM-Sap95), AB074917 (JKK-Sap00), AB161719 (JYW-Sap02), and AB161718 (JTS-Sap02).

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JSM-Sap95  2118 GGTGTCGGGCTTTTCTAGCTGTTTTTCTCCATTCGAGCCCCCCGCTCTGGACTCATCACC  2177
JKK-Sap00       .......................................................C....
JYW-Sap02       .......................................................C....
JTS-Sap02       .......................................................C....
                *******************************************************.****

JSM-Sap95  2178 CCCCGCCGAGGCTGATACACCCGTGGCTGTTGATGTTCCACCCCCAGCTACCTTGACATT  2237
JKK-Sap00       .....................TA.......C.............................
JYW-Sap02       .....................TA.......C...........T.................
JTS-Sap02       .....................TA.......C...........T.................
                *********************..*******.*********** *****************

JSM-Sap95  2238 ACCACAACCTCCGGCTCCTAAACGGGCAGTACCCCCGCAAGATCTTGCCGATGGCGATGT  2298
JKK-Sap00       ...................G........................................
JYW-Sap02       ...................G...............................C........
JTS-Sap02       ...................G........................................
                *******************.*******************************.********
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Fig. 2. Alignment of the region where mutations were most evident. So-called hypervariable region within ORF1 is shown here. Here, also, all the nucleotide substitutions were of transitional type.

hepatitis C virus [8,9]. ORF3 was best conserved among the three ORFs (Fig. 4).

4. Discussion

Spontaneous mutation rate of HEV has not been reported, mainly because there has been no cell culture system that allows persistent infection of HEV and because the natural course of HEV infection in humans and animals is usually a transient one. However, even though HEV does not persist in individual hosts, it persists in community by hopping from host to host, in particular where sporadic hepatitis E is seen frequently, such as Sapporo [2,3,10]. Then, if we trace after the history of evolution of an HEV lineage that has persisted in a community for a considerably long time, it is possible for us to estimate the mutation rate of HEV.

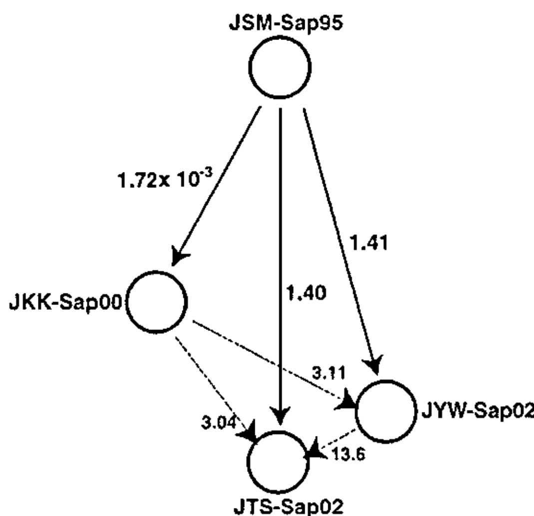

Fig. 3. Mutation rate of HEV in each of the hypothetical evolution pathways. Mutation rate is expressed here as the number of nucleotide substitutions per site per year. Possible pathways are indicated by solid lines, while unlikely pathways by broken lines.

On this rationale, the HEV JSM-Sap95 isolate from a patient living in Sapporo 1995 was assumed in this study to have been successively transmitted from host to host in the community with undergoing spontaneous mutations to become each of the JKK-Sap00, JYW-Sap02, and JTS-Sap02 isolates 5–7 years later. This assumption was supported by the striking resemblance of nucleotide sequences between them, and by the observation that most of the base substitutions among them were found at common positions and were of "transitional" type (Figs. 1 and 2). We then calculated on this assumption the mutation rate of HEV, and it was estimated to be about 1.40×10^{-3} base substitutions per site per year (Fig. 3). Interestingly, this value was very similar to those (1.44×10^{-3} to 1.92×10^{-3}) reported previously for hepatitis C virus (HCV) [8,9], an RNA virus as HEV is.

It is possible that JSM-Sap95 was not the true ancestor of all or any of JKK-Sap00, JYW-Sap02, and JTS-Sap02, but might have been merely a close relative of their true ancestor. Thus, it is most likely that HEV mutates a little slower than the speed estimated in this study. Despite being a preliminary value at present, the mutation rate of HEV

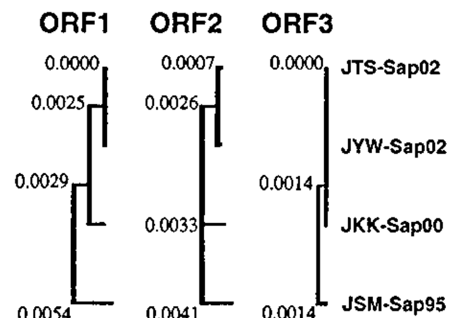

Fig. 4. ORF3 is best conserved. Most likely due to the overlapping with another reading frame, ORF2. UPGMA was used to generate these mini trees.

provided here could be used for molecular clock analyses in order to know when HEV made inroad and began spreading in Japan as apparently indigenous strains. Knowing the time factor may lead to understand how the globalization of HEV occurred: by importation of domestic animals or by human immigrations? To perform the molecular clock analysis for such purposes, however, we need more numbers of complete or semi-complete HEV sequences worldwide.

Acknowledgements

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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.

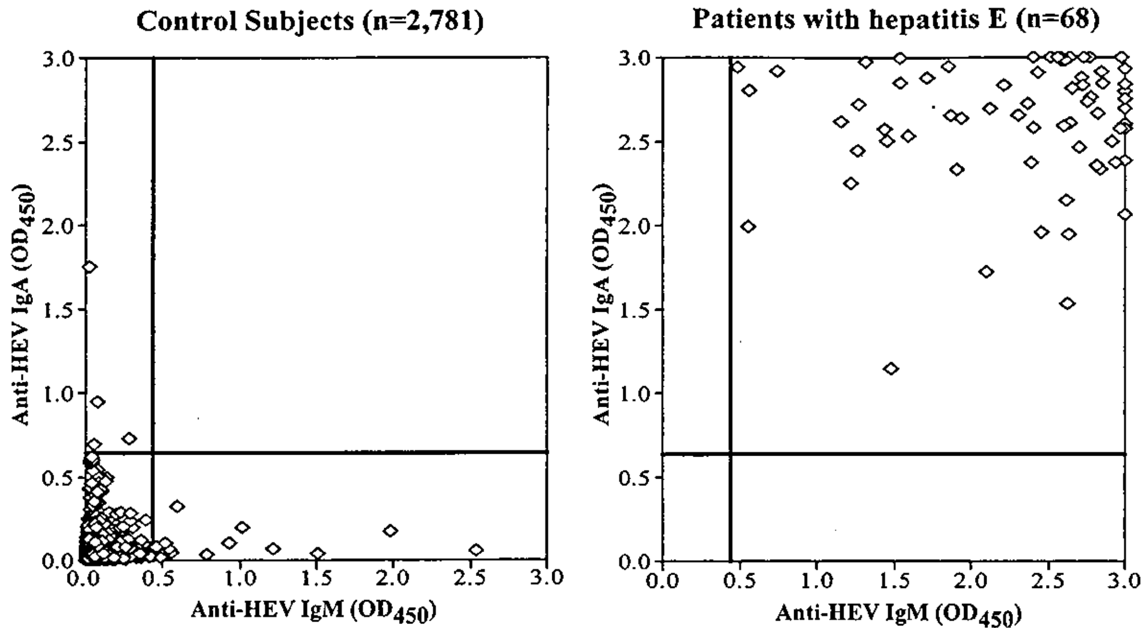

FIG. 1. Distribution of OD values from two ELISAs for anti-HEV IgM and anti-HEV IgA among patients with hepatitis E and among the control subjects. Serum samples from 2,781 subjects who were assumed not to have been infected recently with HEV and from 68 patients with hepatitis E were concurrently tested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

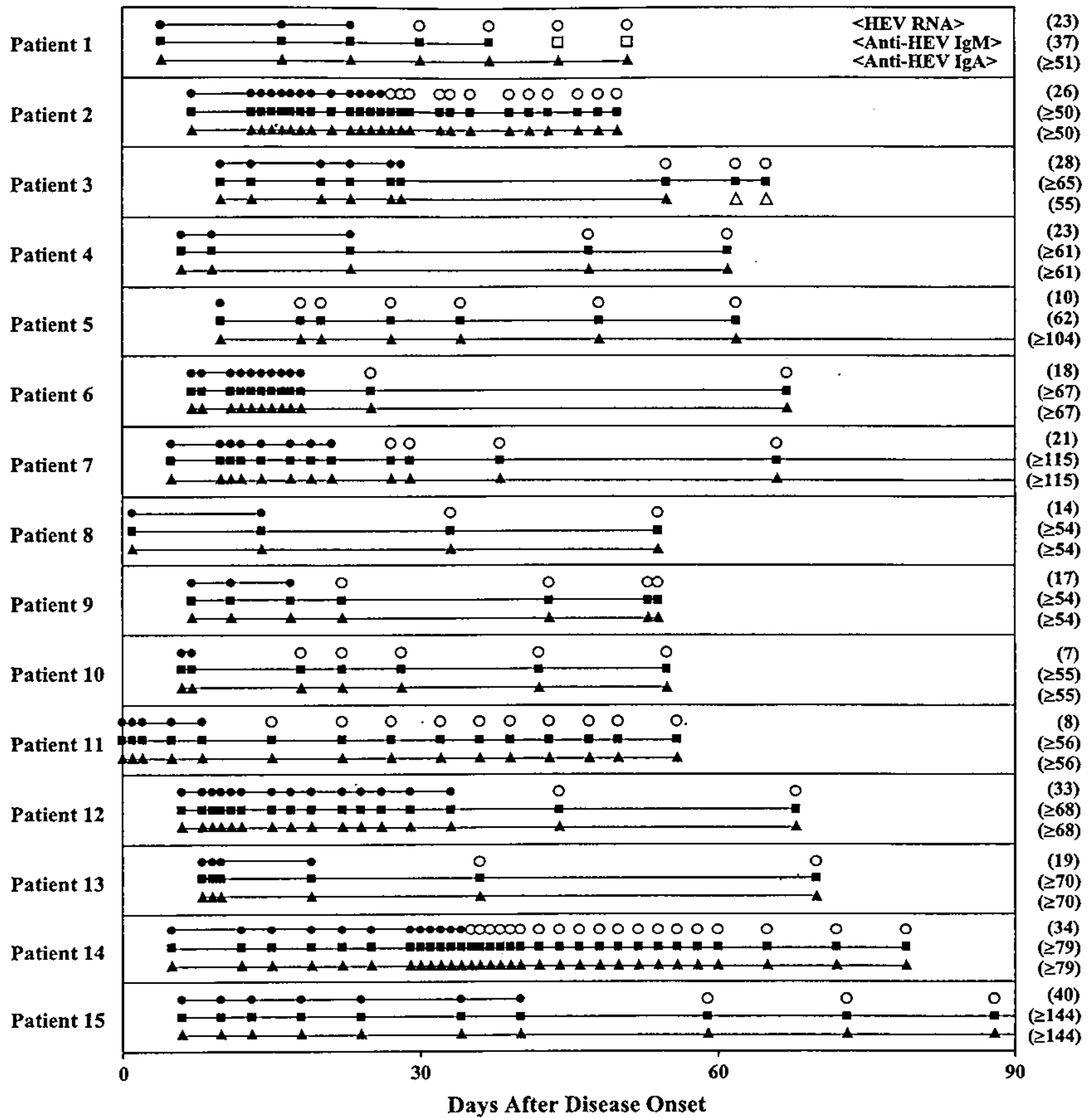

FIG. 2. Detection of HEV RNA, anti-HEV IgM and anti-HEV IgA in initial and follow-up serum samples from 15 patients (patients 1 to 15) with hepatitis E. For each patient, closed and open circles in the top row represent positivity or negativity for HEV RNA, respectively; closed and open boxes in the 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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Challenges in creating a vaccine to prevent hepatitis E

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Summary

Recombinant hepatitis E virus capsid protein (HEV CP) assembles orally immunogenic virus-like particles (VLP) when expressed in an insect cell system. We used plant expression cassettes, pHEV101 and pHEV110, for transformation of potato to express HEV CP, and 10 independent transgenic lines of HEV101 and 6 lines of HEV110 were obtained. ELISA for HEV CP was performed on tuber extracts. Accumulation of HEV CP in tubers varied from about 5 to 30 μg/g fresh tuber depending on the transgenic plant line. We further compared the expression levels with the yield of tubers for each line. Tuber yield varied less than expression levels, and ranged from about 600 to 1000 g per pot. Although Western blot showed that apparently intact HEV CP accumulated, we observed very limited assembly of virus-like particles in potato tubers. Oral immunization of mice with transgenic potatoes failed to elicit detectable anti-CP antibody response in serum, suggesting that VLP assembly is a key factor in orally delivered HEV CP vaccines.
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Keywords: Hepatitis E; HEV; VLP; Transgenic potato

1. Introduction

Hepatitis E virus (HEV) is a causative agent of hepatitis E that occurs in many developing countries [1], and this virus is currently classified into a tentative genus, "Hepatitis E-like viruses." HEV is transmitted mainly by the fecal-oral route, and large epidemics due to this virus are often associated with contaminated water [2,3]. Hepatitis E has been formerly known as an enterically transmitted non-A, non-B hepatitis [4]. The fact that HEV can survive in the intestinal tract suggests that the virus is relatively stable to acid and mild alkaline conditions.

HEV contains a single-stranded positive-sense approximately 7.5 kb RNA molecule that is 3′ polyadenylated and includes three open reading frames (ORFs). ORF1, mapped in the 5′ half of the genome, is thought to encode viral nonstructural proteins. ORF2, located at the 3′-terminus of the genome, encodes a 72 kDa protein for the putative viral capsid. ORF3, with unknown function, is mapped between ORF1 and ORF2 [5]. In the absence of an appropriate cell culture for HEV propagation, research has focused on the expression of the ORF2 protein in heterologous systems. Recently, virus-like particles (VLP) of recombinant hepatitis E virus (rHEV) were produced by using a baculovirus system carrying an N-terminally truncated *ORF2* gene of the Burma strain [6]. Thus, rHEV VLP were formed in Tn5 cells and could be collected from the culture supernatant.

In order to evaluate the potential of rHEV VLP as an oral immunogen, we analyzed the immune responses in mice and monkeys after oral administration [7,8]. The animals were orally inoculated with purified rHEV VLP without adjuvant. ELISA indicated that oral immunization with rHEV VLP induced immune responses in both mice and monkeys. In addition, the monkeys were completely protected from infection when challenge was carried out with native HEV, suggesting that rHEV VLPs are a potential mucosal vaccine for HEV infection.

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For expression and delivery of recombinant subunit vaccine antigens including Norwalk virus capsid protein (NVCP), transgenic plants have been created [9]. Clinical trials using potatoes expressing NVCP showed very promising results, with 19 of 20 volunteers showing immune responses against NVCP delivered by ingestion of raw transgenic potatoes containing approximately 500 μg antigen per dose [10]. Previous studies with NVCP expression in tobacco and potato cells demonstrated that subunits assembled to form VLP very similar to those obtained with baculovirus-infected insect cell expression, although up to 75% of the antigen was present as monomers or partially assembled aggregates [9]. VLP assembly may be important for obtaining stability against acid and protease-mediated degradation in the stomach, as well as for presentation of conformation-dependent epitopes that may be needed for effective virus neutralization.

In this study, we expanded our effort to create transgenic plants that express HEV capsid proteins (HEV CP).

2. Materials and methods

2.1. Preparation and purification of rHEV VLP

The molecular cloning and construction of a recombinant baculovirus Ac5480/7126 harboring the HEV capsid protein gene lacking 111 amino acids at the N-terminal were described previously [6]. The rHEV VLP were prepared using Tn5 cells infected with Ac5480/7126 at a multiplicity of infection of 10. Following 7 days of incubation at 26.5 °C, intact cells and cell debris were removed from the culture medium, and the rHEV VLP were concentrated by centrifugation and purified by isopycnic binding in CsCl gradient. A visible band containing rHEV VLP was collected, and the rHEV VLP were diluted and pelleted by centrifugation.

2.2. Western blot assay

Leaf samples were extracted by FastPrep (speed 5, 30 s) in 4 ml/g leaf of 50 mM sodium phosphate pH 6.6, 50 mM NaCl, 50 mM sodium ascorbate, 1 mM EDTA, 1 mM PMSF, 0.1% Triton X-100, and clarified for 2 min at 4 °C in microcentrifuge. Aliquots were subjected to SDS-PAGE, blotted to PVDF membrane, and probed with guinea pig anti-HEV (1:1000), visualized with ECL+ (anti-guinea pig IgG-HRP 1:5000) on STORM scanner.

2.3. Antigen-capture ELISA

Potato leaf or tuber extracts were prepared as described above in Section 2.2. Microtiter plates were coated with 50 μl per well of rabbit anti-HEV serum diluted 1:10,000 in carbonate/bicarbonate coating buffer overnight at 4 °C. Insect cell-derived HEV VLP reference standard was diluted in PBST/1% dry milk at 100 ng/ml and two-fold dilutions down to 3.125 ng/ml. Leaf or tuber extracts were diluted 25- and 50-fold in PBST/1% dry milk. The reference standards and plant extracts were loaded at 50 μl per well and incubated at 37 °C for 1 h. Wells were washed with PBST and then probed with guinea pig anti-HEV serum diluted 1:5000, followed by goat anti-guinea pig IgG-HRP conjugate (Sigma) diluted 1:5000. Color was developed using TMB substrate solution for 5 min.

2.4. Construction of plant expression vector

Intermediate plant expression cassettes were constructed using a vector pIBT210 [11]. Since two truncated forms

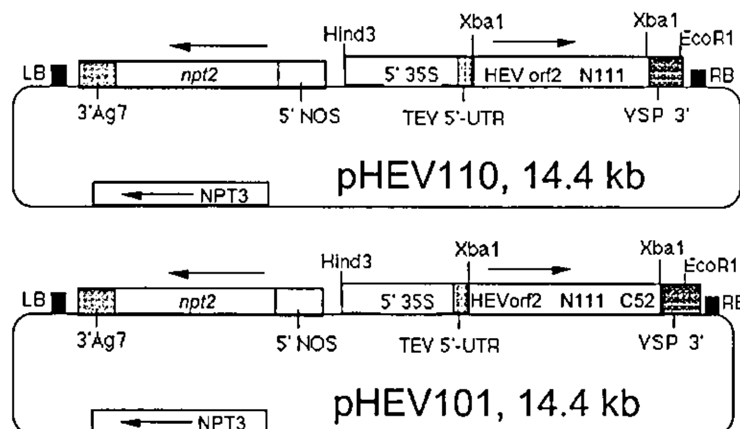

Fig. 1. Plant transformation vectors for expression of HEV CP. Binary T-DNA plasmid vectors, pHEV101 and pHEV110, for expression of HEV CP, p54 and p58, are shown. They contain left border (LB) and right border (RB) sequence motifs that delineate the DNA to be transferred (T-DNA) and integrated into nuclear chromosomal DNA. Within the T-DNA borders lies a selectable marker (*ntp2*), which confers resistance to the antibiotic kanamycin, and will allow specific regeneration of transformed plants. Also included are expression cassettes for HEV CP, which are driven by the constitutive CaMV 35S promoter linked to the tobacco etch virus (TEV) 5′-UTR, which acts as a translational enhancer, and terminated by the soybean VSP 3′ end [9].

of HEV ORF2 appeared to yield VLP assembly in insect cells (ΔN111 and ΔN111/ΔC52), we inserted these into pIBT210. Then, the expression cassettes were transferred into a binary vector (pGPTV-Kan) for use in *Agrobacterium*-mediated delivery of foreign DNA into plant cells. These constructs, pHEV101 (ΔN111/ΔC52) and pHEV110 (ΔN111), are shown in Fig. 1.

3. Results

3.1. Characterization of rHEV

The capsid proteins of HEV with its N-terminal 111 amino acids truncated were expressed with a recombinant baculovirus in insect cells, where the capsid proteins self-assembled into VLP [6]. The rHEV VLP were purified by centrifugation and characterized by SDS-PAGE and Western blot assay, where a major protein band with a molecular weight of 54 kDa was observed. The particles possess antigenicity similar to that of authentic HEV particles and consequently they appear to be a good antigen for the sensitive detection of HEV-specific IgG and IgM antibodies [12]. Furthermore, the VLP may be the most promising candidate for an HEV vaccine owing to its potent immunogenicity [7,8]. Therefore, we used the same construct to express HEV CP in the transgenic plant.

3.2. Coding sequence analysis

We first examined the coding sequence for the HEV CP to determine whether the nucleotide sequence should be altered for optimization of plant expression. Codon use is fairly favorable to both dicot and monocot plants. Of 660 total codons, 3.6% are monocot-unfavorable and 12.8% are dicot-unfavorable, defined as either making up less than 10% of codon choice for that amino acid or less than one third the frequency of the most popular codon for that amino acid, inclusive.

3.3. Expression in potato plants

We used pHEV101 and pHEV110 for transformation of potato "Desiree" as described [9,13]. After regeneration of multiple independent kanamycin-resistant lines, we screened leaf samples by ELISA for HEV CP expression. Expression levels ranged up to approximately 0.33% total soluble protein, which is similar to the levels we obtained for NVCP [9]. There was no apparent difference in expression from either construct pHEV101 (ΔN111/ΔC52 coding sequence) and pHEV110 (ΔN111 coding sequence) as the range and maximal expression were similar for both.

We selected the best lines for transplant to the greenhouse and after 2 months growth we assayed leaves for expression of HEV CP by ELISA. We observed that the antigen

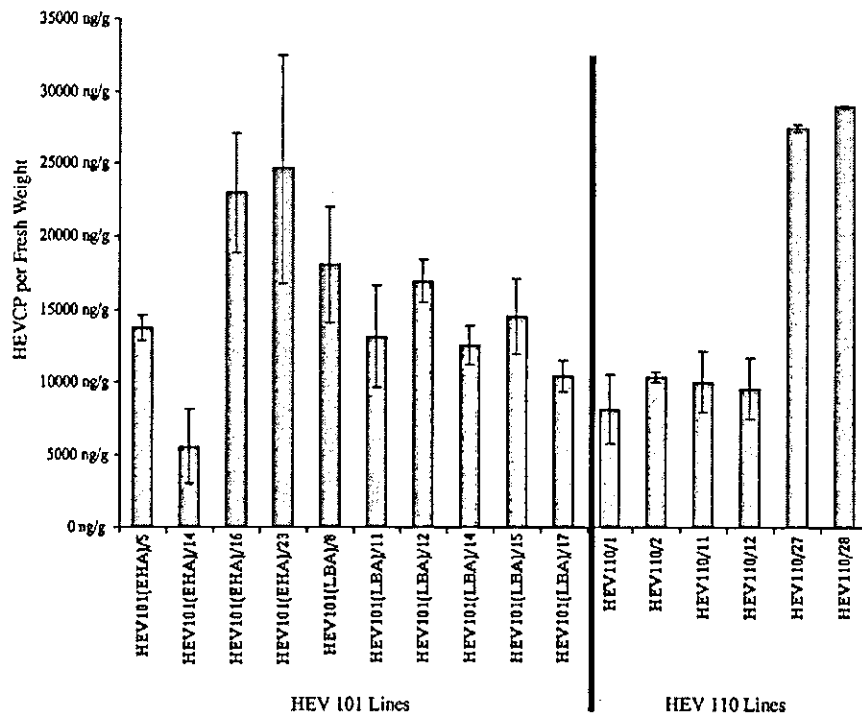

Fig. 2. Expression of HEV CP in tubers of transgenic potato lines. HEV101 (10 lines) or HEV110 (6 lines) tubers were extracted and assayed by ELISA for HEV CP. Error bars indicate standard error for three different tubers from the same line.

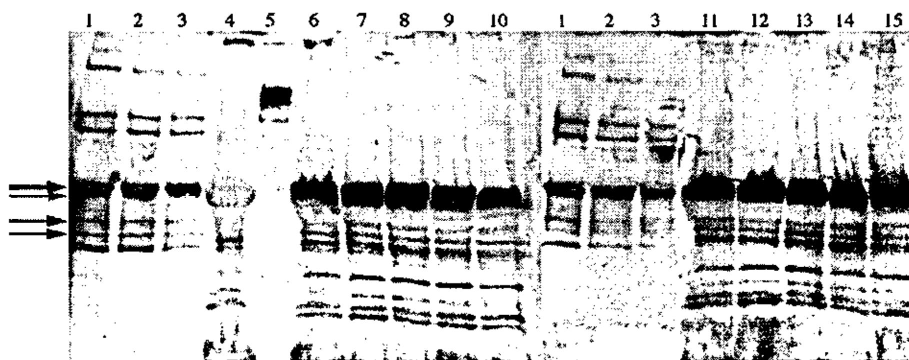

Fig. 3. Western blot of HEV CP expressed in potato leaves. Leaf samples were extracted by FastPrep and clarified by centrifugation. Aliquots were subjected to SDS-PAGE, blotted to PVDF membrane, and probed with guinea pig anti-HEV, visualized with ECL+ on STORM scanner. Lanes are: (1) 50 ng HEV VLP from baculovirus-infected insect cells, (2) 25 ng HEV VLP, (3) 12.5 ng HEV VLP, (4) untransformed Desirée extract, (5) BioRad Kaleidoscope Molecular Weight Marker, (6) HEV101(EHA)/5, (7) HEV101(EHA)/12, (8) HEV101(EHA)/16, (9) HEV101(EHA)/17, (10) HEV101(LBA)/11, (11) HEV101(LBA)/14, (12) HEV110/1, (13) HEV110/2, (14) HEV110/11 and (15) HEV110/12. Bold arrow indicates major band of VLP sample that corresponds to the 54 kDa HEV CP; thin arrows indicate products of proteolysis.

expression on a total protein basis was reduced four- to five-fold compared to that in tissue-cultured plantlets. This is not unexpected, since soil-grown plants in natural light have higher levels of total leaf protein. Further, it is possible that the recombinant antigen is less stable in the soil-grown plants.

3.4. HEV CP expression in potato tubers

Ten independent transgenic lines of HEV101 and six lines of HEV110 were grown to maturity in the greenhouse and tubers were harvested and washed. ELISA for HEV CP was performed on tuber extracts as described for leaf extracts. Accumulation of HEV CP in tubers varied from about 5 to 30 μg/g fresh tuber, depending on the transgenic plant line (Fig. 2). This compares well with the expression of NVCP in potato tubers [9] and is better than expression of *E. coli* LT-B protein in potato [13]. We further compared the expression levels with the yield of tubers for each line. Tuber yield varied less than expression levels, and ranged from about 600 to 1000 g per pot. We used these data to select lines HEV101-16, HEV101-23, HEV110-27, and HEV110-28 for highest yields of recombinant protein.

Western blot of transgenic potato leaf extract showed that most of the recombinant HEV CP accumulated as 54 kDa, similar to the insect cell-derived antigen (Fig. 3). Some apparent proteolytic products of lower M_r were observed in both insect cell- and potato-derived material. HEV CP in plants transformed with either HEV101 or HEV110 showed similar patterns, with no qualitative or quantitative differences apparent. Failure to detect a larger protein for the single-truncation HEV101 (ΔN111) than that observed for the double truncation HEV110 (ΔN111/ΔC52) suggests that the ΔC52 truncation may occur in planta via an endogenous protease.

4. Discussion

HEV CP has been expressed in baculovirus-infected insect cell system and shown to assemble VLP [6,14]. The VLP have several advantages for the mucosal immunogen as follows: (1) rHEV VLP are composed of a single protein assembled into particles without nucleic acid. (2) rHEV VLP are easy to prepare and purify in a large quantities, approximately 1 mg per 2×10^7 insect cells. (3) rHEV VLP are antigenically similar to the native virion. (4) rHEV VLP are highly immunogenic in experimental animals when injected parenterally.

Our goal is to create transgenic plants that express HEV CP in edible tissues as VLP, in order to obtain an economical oral vaccine. It is likely that the success of oral delivery using VLP from insect cells is due to the particulate structure of the antigen, which contributed either to enhanced resistance to degradation in the gut or to enhanced uptake into the gut immune system.

In our studies with potato expressing HEV CP, we found very few VLPs, with the great majority of ELISA-positive antigen remaining near the top of a sucrose gradient (data not shown). Oral immunization of mice with potatoes expressing HEV CP failed to elicit detectable antibody responses in serum (data not shown). We extracted fecal pellets on day 18 after oral immunization on day 17 to evaluate the content of ELISA-reactive HEV capsid protein. Substantial antigen (3–4% of the dose) was present in pellets of mice that were fed HEV CP potato or gavaged with insect cell-derived VLP. Thus, the potato cells probably provided some protection to the soluble HEV CP present in potato tubers, and perhaps even limited uptake of antigen that may have been present as VLP. Since orally delivered insect cell-derived VLPs stimulated antibody responses and protected monkeys against HEV challenge [8], it is likely that poor VLP assembly in potato

was a major factor in the lack of oral immunogenicity of potato-derived HEV CP in mice. Future studies should focus on the optimization of VLP assembly in plant tissues, which may involve alternative plant host systems, and/or tissue and subcellular targeting of antigen.

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RESEARCH ARTICLE

DNA vaccine-encapsulated virus-like particles derived from an orally transmissible virus stimulate mucosal and systemic immune responses by oral administration

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Delivery of foreign genes to the digestive tract mucosa by oral administration of nonreplicating gene transfer vectors would be a very useful method for vaccination and gene therapy. However, there have been few reports on suitable vectors. In the present study, we found that plasmid DNA can be packaged in vitro into a virus-like particle (VLP) composed of open reading frame 2 of hepatitis E virus, which is an orally transmissible virus, and that these VLPs can deliver this foreign DNA to the intestinal mucosa in vivo. The delivery of plasmid DNA to the mucosa of the small intestine was confirmed by the results of immunohistochemical analyses using an expression plasmid encoding human immunodeficiency virus env (HIV env) gp120. After oral administration of VLPs loaded with HIV env cDNA, significant levels of specific IgG and IgA to HIV env in fecal extracts and sera were found. Moreover, mice used in this study exhibited cytotoxic T-lymphocyte responses specific to HIV env in the spleen, Payer's patches and mesenteric lymph nodes. These findings suggest that VLPs derived from orally transmissible viruses can be used as vectors for delivery of genes to mucosal tissue by oral administration for the purpose of DNA vaccination and gene therapy.

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Keywords: *VLP; oral DNA vaccine; CTL; HIV; mucosal immunity*

Introduction

The successful outcome of novel gene therapies and DNA vaccinations largely depends on the development of effective delivery systems.[1] In human applications, both the efficacy and safety of any delivery system used for gene transfer are major concerns. It has been shown that tissue-specific gene transfer by a viral vector could be achieved naturally and effectively through cell specificity of the virus receptors.[2] However, there is a risk of vector toxicity through viral infection of the host cells. Also, the limited sizes of transgenes often present a serious obstacle. Nonviral vectors, such as liposomes, are safer but do not have a cell-specific targeting component and have limited transduction both *in vitro* and *in vivo*. This limitation has been partly overcome by the development of molecular conjugates consisting of cell-specific ligands that confer cell specificity to nonviral vectors.[3,4]

The development of a system for delivering genes to or conferring immunity to mucosal tissue by oral administration would provide a convenient means for effective treatment or prevention of various human diseases, including cancers, infectious diseases and immunological disorders.[5] Since many pathogenic viruses and bacteria establish their initial infections through the mucosal surface, vaccine strategies that can stimulate mucosal immunity have been widely studied (reviewed in Ogra *et al*[6]). However, there are several difficulties in oral immunization with nonreplicating molecules, such as low pH in the stomach, the presence of proteolytic enzymes in the digestive tract and the presence of physical as well as biochemical barriers associated with the mucosal surface itself.[6]

Among the various nonreplicating molecules, a virus-like particle (VLP), an empty particle with a structure similar to that of an authentic virus particle, offers the possibility of a new approach for vaccine development.[7] It is expected that the VLP structure will provide resistance to severe environments in the digestive tracts and enable specific binding to the mucosal surface if an appropriate VLP is chosen.[8] However, VLPs can induce immune responses to themselves, and this is a problem for using VLPs as a vaccine vector to carry foreign DNA. A system using polyoma virus VP1 VLPs as a carrier of DNA by intranasal administration has been reported.[9] These VLPs work as an adjuvant, since DNA vaccine can induce immune responses by intranasal administration without VLPs. Hepatitis E virus (HEV) is an unclassified calicivirus-like, positive-strand RNA virus that causes

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