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Application of Truncated Immunodominant Polypeptide from Hepatitis E Virus (HEV) ORF2 in an Assay To Exclude Nonspecific Binding in Detecting Anti-HEV Immunoglobulin M▽

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The diagnosis of recent hepatitis E virus (HEV) infection depends on serologic testing for anti-HEV IgM; however, false-positive results may occur. In the present study, we cloned the ORF2 fragment of genotype 4 HEV and demonstrated that a subregion covering amino acids 459 to 607 in ORF2 forms the immunodominant B-cell epitopes, as it does in genotype 1 viruses. Truncation of several residues from either the N or C terminus of the polypeptide abolished the reactivity of anti-HEV from naturally infected persons. By the combination of high reactivity of the immunodominant polypeptide and poor reactivity of the truncated polypeptide, we established an indirect enzyme-linked immunosorbent assay (ELISA) to detect anti-HEV IgM. In this assay, all 37 sera that were HEV RNA positive reacted with the immunodominant polypeptide but not with the truncated one, and none of 159 sera from healthy persons reacted with either of the polypeptides. In retesting of 117 sera that originally tested positive for anti-HEV IgM, using a Genelabs kit, only 34 were positive and 83 were negative. Western blot analyses and other experiments strongly indicated that these 83 discordant sera were negative for anti-HEV IgM. Furthermore, among the 117 sera, 5 reacted with both the immunodominant and truncated polypeptides, with comparable optical densities at 450 nm. However, their reactivity was demonstrated to result from nonspecific binding. Together, the data indicate that the poor reactivity of a truncated ORF2 polypeptide can be used to exclude nonspecific binding in the detection of anti-HEV IgM.

Hepatitis E virus (HEV), transmitted by the fecal-oral route, is the main cause of acute self-limiting hepatitis in many developing countries (1, 2, 19). Recently, HEV infection and the disease hepatitis E have also attained attention in industrialized countries (3, 14). HEV is a single-stranded positive-sense RNA virus with a genome of approximately 7.2 kb. The viral genome contains three open reading frames (ORFs). There are four major genotypes of HEV identified, yet there is only a single serotype (19). In China, most sporadic cases are caused by genotype 4 (7, 13).

Clinically, hepatitis E is not distinguishable from acute hepatitis caused by other viruses. Thus, the diagnosis of hepatitis E depends upon laboratory evidence of recent infection. Detection of HEV RNA in serum or feces by reverse transcription (RT) and nested or real-time PCR is direct evidence of acute infection; however, in addition to the expensiveness of the assays, viremia usually exists at the early acute phase and subsides soon after the apparent symptoms. Thus, the approach of detecting HEV RNA is not a practical way to clinically diagnose hepatitis E.

Detection of IgM antibody against HEV (anti-HEV IgM) in serum can diagnose acute HEV infection. Commercial enzyme-linked immunosorbent assay (ELISA) kits for IgM are available; these assays were established based on recombinant antigens and/or synthetic polypeptides derived from ORF2 and/or ORF3 sequences (4, 6, 26, 28). However, false-positive results occurred when the available kits were used to detect anti-HEV IgM (5, 8, 9, 16, 18). Therefore, a more specific assay for anti-HEV IgM that does not sacrifice sensitivity would be valuable for diagnosing hepatitis E.

The polypeptide encoded by ORF2 forms the principal (probably only) structural protein of HEV. It has been documented that the C-terminal portion of the ORF2 polypeptide covers the immunodominant epitopes (17, 20), which are located in amino acids (aa) 459 to 607 (29). A polypeptide in which several aa residues are truncated from either terminus does not react with the anti-HEV induced by natural infection in humans and experimental infection in nonhuman primates (29). Based on these observations, we assumed that the truncated ORF2 polypeptide could serve as a "nonreactive polypeptide" to rule out false-positive results in the detection of anti-HEV IgM. Here we present data to support our assumption that false-positive results may be excluded by use of the truncated polypeptide.

MATERIALS AND METHODS

Serum samples. In total, 313 serum samples were included in the present study and were divided into three groups. One group comprised the sera from 159 healthy persons who were negative for anti-HEV IgM, as detected with a Genelabs kit (Genelabs Diagnostics Pte. Ltd., Singapore Science Park, Singa-

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TABLE 1. Primers for cloning and expression of HEV ORF2 gene fragments

Primer	Sequence (5′–3′)
HEV-ORF2-U1stF	CCGACAGAATTGATTTCGTC
HEV-ORF2-U1stR	TTACCYACCTTCATYTTAAG
HEV-ORF2-U2ndF	AAYGCTCAGCAGGAYAAGGG
HEV-ORF2-U2ndR	CAYTCHGGGCARAARTCATC
HEV-ORF2-PstI-F459	AACTGCAGTCTCGTCCTTTTT CTGTGCT[a]
HEV-ORF2-PstI-F472	AACTGCAGTGGCTTTCACTTA CGGCTGC[a]
HEV-ORF2-EcoRI-R594	TTGAATTCAGAAACAGGCCC GGAACCCA[b]
HEV-ORF2-EcoRI-R607	TTGAATTCCAATGCCGAATGG GGTGCGA[b]

[a] Underlined sequences are PstI sites.
[b] Underlined sequences are EcoRI sites.

pore), and negative for IgM antibodies against hepatitis A virus and hepatitis B virus core antigen and for IgG antibody against hepatitis C virus. The second group was composed of samples from 37 acute hepatitis E patients who were diagnosed by the detection of HEV RNA (7). The third group included 117 sera which were positive for anti-HEV IgM, repeatedly detected with a Genelabs kit. All sera were stored at −70°C. This study was approved by the institutional review boards of the Nanjing Drum Tower Hospital.

Cloning and expression of HEV ORF2 fragment. Total RNA was extracted from 200 μl serum from a hepatitis E patient with positive anti-HEV IgM, using Trizol (Invitrogen, CA). The ORF2 fragment was cloned by RT-PCR. The cloning primers were U1stF and U1stR as the outer pair and U2ndF and U2ndR as the inner pair (Table 1). The purified PCR products were inserted into T-tailed pGEM-T vectors (Promega, WI). Genotypes were determined by DNA sequencing.

Three fragments of different sizes, covering aa 459 to 607, 472 to 607, and 459 to 594 (residue positions refer to the prototype HEV sequence [GenBank accession number AF444003]) in ORF2, were amplified by PCR with primers containing a PstI or EcoRI site (Table 1). The enzyme-digested PCR products were ligated to the expression vector pRSET-C (Invitrogen), which has a sequence coding for six histidine residues (His_6) at the N terminus of the recombinant polypeptide for purification. The inserts in the plasmids isolated from single colonies were sequenced. *Escherichia coli* BL21(DE3) (Invitrogen) was transformed with each of the ORF2 recombinant plasmids, carrying the above three ORF2 fragments, cultured in LB overnight, and then subcultured in super optimal broth (SOB) at 37°C for the expression of ORF2. Bacterial pellets were subjected to SDS-PAGE to examine the polypeptides.

The recombinant polypeptides were purified by nickel-chelation affinity chromatography via the His_6 tag. Briefly, the bacterial pellets were lysed by sonication in guanidinium lysis buffer (6 M guanidine-HCl, 100 mM NaH_2PO_4, 10 mM Tris-Cl, pH 8.0). The polypeptides bound to the nickel were eluted with denaturing elution buffer (8 M urea, 100 mM NaH_2PO_4, 10 mM Tris-Cl, pH 3.5). Each polypeptide was checked by SDS-PAGE, and the concentration was determined with a bicinchoninic acid (BCA) assay (Pierce, IL). Prior to use in ELISA, the polypeptides were diluted with carbonate-bicarbonate buffer (pH 9.6) for renaturation.

Western blot analysis of anti-HEV in patient sera. The recombinant ORF2 polypeptides containing aa 459 to 607, 472 to 607, and 459 to 594 were separated by 12% SDS-PAGE and transferred to a polyvinylidene difluoride (PVDF) membrane. The membranes were blocked with 5% skim milk in 0.05% Tween 20 in phosphate-buffered saline (PBS-T) at room temperature for 1 h and then sliced into three samples. The first was incubated with peroxidase-labeled anti-His_6 (1:1,000 dilution) for 1 h. The others were incubated for 3 h with serum samples diluted 100-fold in 5% skim milk. After four washes, horseradish peroxidase (HRP)-conjugated anti-human IgG or IgM (1:2,000) was incubated with the membranes for 1 h to detect the bound antibodies. The immunoreactivity was detected using a DAB enhanced liquid substrate system (Sigma, MO).

Enzyme-linked immunosorbent assay. An indirect ELISA was developed to detect anti-HEV IgG and IgM in sera of patients. ELISA plate wells were separately coated with 100 μl purified ORF2 polypeptides 459-607 and 472-607, diluted to 1 μg/ml in carbonate-bicarbonate buffer (pH 9.6), overnight at 4°C. The wells were washed twice with PBS-T and then blocked with 300 μl 5% skim milk in PBS-T at 37°C for 1 h. After three washes, 100 μl serum diluted 100-fold in 5% skim milk was added to the plates, followed by incubation at 37°C for 1 h. After another five washes, 100 μl HRP-conjugated anti-human IgG or IgM (Sigma), diluted 2,000-fold in PBS-T, was added to detect the bound antibodies. Following incubation at 37°C for 0.5 h, the plates were washed as described above, and 100 μl of tetramethylbenzidine solution (Sigma) was added to the wells. After incubation at room temperature for 15 min, the color development was stopped by adding 50 μl of 2 M H_2SO_4. The optical density at 450 nm (OD_{450}) was determined on a microplate reader (Bio-Rad, CA). The OD_{450} in the well coated with the immunodominant polypeptide (aa 459 to 607) of ORF2 was denoted OD_{IP}, and that in the well coated with the truncated immunodominant polypeptide (aa 472 to 607) was designated OD_{TP}.

Anti-HEV IgM detected by commercially available kits. Commercially available ELISA kits for anti-HEV IgM were obtained from Genelabs (Genelabs Diagnostics Pte. Ltd., Singapore Science Park, Singapore) and Wantai (Wantai Biological Pharmacy Enterprise Co., Ltd., Beijing, China). The Genelabs kit is an indirect ELISA based on recombinant and synthetic peptides derived from ORF2 and ORF3 of genotypes 1 and 2 (6, 26). The Wantai kit is a capture ELISA, in which anti-human IgM (μ chain specific) is used to coat the plate and HRP-conjugated ORF2 polypeptide (genotype 1) is applied to detect the bound anti-HEV IgM; the polypeptide covers aa 394 to 606 in ORF2 (4, 28). The cutoff values were calculated per the manufacturers' instructions.

RESULTS

Production of recombinant ORF2 polypeptides. To obtain the HEV ORF2 gene, we amplified the fragment of interest, using RT-PCR, from four sera collected from hepatitis E patients positive for anti-HEV IgM (detected with a Genelabs kit). One of the sera was positive, and the resultant products were inserted into the pGEM-T vector and sequenced. BLAST searching in GenBank showed that the amplified sequence had the highest similarity (95.4% identity) to the corresponding region of an HEV (genotype 4) previously isolated in China (GenBank accession number EF077630). Thus, we cloned a genotype 4 ORF2 fragment.

The ORF2 protein of HEV genotype 1 has been characterized extensively through use of the recombinant polypeptide (10, 11). It may form homodimers and even higher-order oligomers (15, 23, 27), and the C-terminal portion is critical for dimerization (15, 27). The C-terminal portion covering aa 459 to 607 constitutes the neutralization and immunodominant epitopes (29, 30). However, the ORF2 polypeptide of genotype 4 is less well studied. To characterize the ORF2 polypeptide of genotype 4, we produced the corresponding ORF2 polypeptide and N- and C-terminally truncated polypeptides in *E. coli*, as previously described (29). Although the ORF2 polypeptide of HEV genotype 4 is putatively considered to have an additional 11 to 14 residues at the N terminus (22, 24, 25), we designated the aa positions according to the sequence of genotype 1 so that it is convenient to make comparisons between the different genotypes.

Figure 1A shows that three polypeptides, covering aa 459 to 607, 472 to 607, and 459 to 594, were all produced in *E. coli*. After purification with a nickel-affinity column, each polypeptide showed a single band in SDS-PAGE when the sample was heated at 95°C for 5 min in Laemmli buffer (Fig. 1B). Approximately 3 mg of each purified polypeptide was obtained from 100 ml of bacterial culture.

Dimeric characterization of ORF2 polypeptide. Previous studies have shown that the genotype 1 ORF2 polypeptide forms homodimers and that the C-terminal portion is responsible for dimerization (15, 27). To observe whether the dimeric property is common in the genotype 4 ORF2 polypeptide, we analyzed the three polypeptides, 459-607, 472-607, and 459-

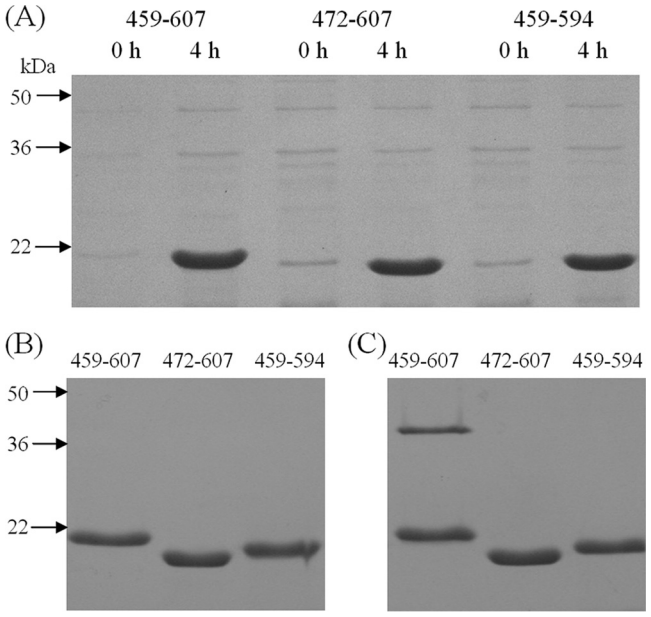

FIG. 1. Expression and purification of histidine-tagged HEV ORF2 polypeptides in *E. coli*. (A) SDS-PAGE of polypeptides spanning amino acids 459 to 607, 472 to 607, and 459 to 594. 0 h and 4 h, 0 and 4 h after adding IPTG (isopropyl-β-D-thiogalactopyranoside). (B) Standard SDS-PAGE of purified polypeptides. (C) SDS-PAGE of purified polypeptides, in Laemmli buffer, which were not heated.

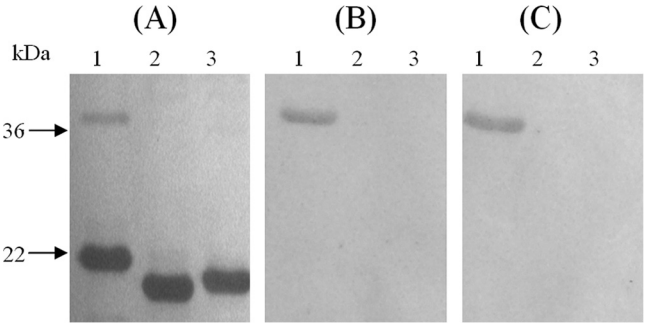

FIG. 2. Antigenic characterization of ORF2 polypeptides by Western blot analysis. His_6-tagged polypeptides in Laemmli buffer were not heated before being run in the gel. Lanes 1 to 3 indicate the polypeptides covering amino acids 459 to 607, 472 to 607, and 459 to 594 in ORF2, respectively. The color was developed by the DAB enhanced liquid substrate system. (A) The membrane was probed with peroxidase-labeled anti-His_6. (B and C) The membranes were incubated with the serum from a hepatitis E patient and then probed with peroxidase-labeled anti-human IgG (B) and IgM (C).

594, by SDS-PAGE under different conditions. When polypeptide 459-607 was heated in Laemmli buffer, it showed a single band at the expected size of 21 kDa (Fig. 1B); however, when the polypeptide was not heated, it presented a band of the expected size (21 kDa) as well as another band corresponding to a high-molecular-mass polypeptide (42 kDa) (Fig. 1C), indicating that this band was the homodimer. On the other hand, polypeptides 459-594 and 472-607 showed single bands at the positions of the expected sizes, regardless of whether they were heated or not heated (Fig. 1B and C), suggesting that the truncated polypeptides did not form dimers. Thus, the dimerization of genotype 4 ORF2 requires aa 459 to 607 in the ORF2 protein, which is generally in accordance with the region of the genotype 1 polypeptide reported previously (15).

Characterization of antigenicity of ORF2 polypeptide. Since polypeptide 459-607 presented both monomers and dimers when it was not heated (Fig. 1C), we attempted to see whether anti-HEV from hepatitis E patients reacted with the dimeric or monomeric form or with both. After the polypeptides were transferred to the membrane, anti-HEV serum was added and then the blot was probed with enzyme-conjugated anti-human IgG and IgM. As controls, Fig. 2A presents the reactivities of anti-His_6 to the recombinant polypeptides, since each of them had a His_6 tag at the N terminus. Figures 2B and C show that both anti-HEV IgM and IgG naturally produced in the patients reacted only with the dimer, not with the monomer, of polypeptide 459-607. Furthermore, anti-HEV did not react with polypeptides 472-607 and 459-594, which did not form dimers, as demonstrated in Fig. 1. We tested 13 sera from acute hepatitis E patients, and all showed the same results. Thus, dimerization of the polypeptide spanning aa 459 to 607 in ORF2 is essential for formation of the epitopes recognized by natural infection-induced anti-HEV directed against this region.

We further analyzed the antigenicity of these polypeptides under natural conditions by using ELISA. We developed an indirect ELISA based on polypeptides 459-607 and 472-607 to observe the immunoreactivities of anti-HEV IgG and IgM, respectively. All 37 sera positive for HEV RNA reacted with polypeptide 459-607 but not with polypeptide 472-607 in the test for both anti-HEV IgG and IgM. Table 2 shows the representative OD_{450} values for 10 samples. The data also demonstrated that anti-HEV IgG and IgM reacted only with polypeptide 459-607, not with polypeptide 472-607, under natural conditions, in agreement with the results of Western blot analyses.

Exclusion of false-positive anti-HEV IgM results by use of polypeptide 472-607. Based on the poor reactivity of anti-HEV to polypeptide 472-607, we assumed that the truncated polypeptide may serve as a "negative polypeptide" to rule out nonspecific binding in the detection of anti-HEV IgM. We used the indirect ELISA to measure anti-HEV IgG and IgM in the 159 sera that were negative for anti-HEV IgM by a Genelabs kit. None of them reacted with either polypeptide in the detection of IgM; however, in the detection of IgG, 19 (11.9%) sera reacted with polypeptide 459-607 but not with polypeptide 472-609. Representative results are shown in Table 2. To set the cutoff value for anti-HEV IgM in the ELISA based on polypeptide 459-607, we chose twice the average plus 2 standard deviations of the OD_{450} values of the negative-control samples. Statistically, the mean OD_{450} value for the 159 negative samples was 0.172 ± 0.027, and thus, a sample with an OD_{450} of >0.400 was preliminarily judged to be immunoreactive. Since all sera positive for HEV RNA reacted with the immunodominant polypeptide but not with the truncated polypeptide (Fig. 2 and Table 2), we applied the truncated polypeptide as an "irrelevant polypeptide" in the ELISA. As a complementary criterion, a serum with a ratio of OD_{IP} to OD_{TP} of >2 was considered to be specifically immunoreactive, as described elsewhere (12); when the ratio was <2, a value for OD_{IP} minus OD_{TP} of >0.5 was also considered to indicate a specifically immunoreactive serum.

TABLE 2. Detection of anti-HEV IgM and IgG with indirect ELISA based on immunodominant and truncated polypeptides

Sample	OD_{450}			
	IgG ELISA[a]		IgM ELISA[a]	
	Polypeptide 459-607	Polypeptide 472-607	Polypeptide 459-607	Polypeptide 472-607
HEV patients[b]				
H01	1.661	0.214	1.237	0.157
H02	1.641	0.257	1.19	0.164
H03	1.509	0.103	1.243	0.198
H04	1.936	0.104	1.834	0.228
H05	1.666	0.094	1.635	0.171
H06	1.108	0.131	1.437	0.231
H07	1.882	0.146	1.778	0.122
H08	1.514	0.104	1.726	0.071
H09	1.834	0.116	1.505	0.228
H10	1.004	0.1	1.172	0.23
Healthy persons[c]				
N01	0.13	0.112	0.2	0.191
N02	0.171	0.142	0.303	0.259
N03	0.216	0.182	0.193	0.143
N04	0.272	0.247	0.136	0.109
N05	1.48	0.167	0.171	0.152
N06	0.155	0.138	0.17	0.156
N07	0.293	0.233	0.225	0.136
N08	1.116	0.197	0.175	0.164
N09	0.103	0.101	0.141	0.141
N10	0.264	0.262	0.335	0.321

[a] Microplate wells were coated with the immunodominant ORF2 polypeptide, covering amino acids 459 to 607, or the truncated polypeptide, covering amino acids 472 to 607.
[b] Patients were positive for HEV RNA by RT-PCR.
[c] Persons were negative for anti-HEV IgM, as detected with a Genelabs kit.

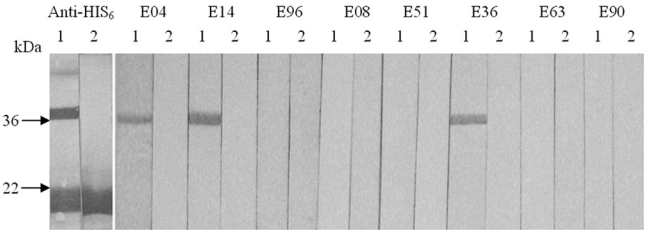

FIG. 3. Western blot analysis of eight sera that reacted with both immunodominant and truncated ORF2 polypeptides. His_6-tagged polypeptides in Laemmli buffer were not heated before being run in the gel. Lanes 1 and 2, histidine-tagged polypeptides covering aa 459 to 607 and aa 472 to 607 in ORF2, respectively. Anti-His_6, the membranes were probed with peroxidase-labeled anti-His_6; E04 to E90, the membranes were incubated with each of the sera and then probed with peroxidase-labeled anti-human IgM. The color was developed by the DAB enhanced liquid substrate system.

We used the ELISA developed in this study to retest anti-HEV IgM in 117 serum samples which were positive for anti-HEV IgM, as detected by a Genelabs kit. Surprisingly, only 34 serum samples were positive for IgM, and the other 83 samples were negative. Since the assay developed in this study was an indirect ELISA and the Genelabs kit is also an indirect ELISA, the high proportion of discrepancy led us to retest these samples with an assay based on different principles. Thus, we measured anti-HEV IgM in these 117 sera with a Wantai ELISA kit and found that 35 of them were positive and the other 82 were negative. There were 32 samples which showed positive results and 80 samples which were negative in both the ELISA in this study and the Wantai ELISA kit.

To clarify whether the sera which had discordant results in different assays were positive or negative for anti-HEV IgM, we performed Western blotting to analyze the reactivities of 43 discordant sera and 14 dual-positive sera to polypeptides 459-607 and 472-607. While all dual-positive sera reacted with the dimer of polypeptide 459-607, none of the discordant sera showed a positive reaction (data not shown). Additionally, we checked the alanine aminotransferase (ALT) levels of these patients and found no patients with abnormal results (data not shown). Together, these data strongly suggest that the positive results for discordant samples detected by the Genelabs kit were caused by nonspecific binding.

In measuring the IgM level in 117 sera with the ELISA used in this study, we also found that eight sera had higher OD values (OD_{450} of >0.4) in the plate wells coated with polypeptide 459-607 and polypeptide 472-607 (Table 3); however, only three, E4, E14, and E96, had an OD_{IP} markedly higher than the OD_{TP} (OD_{IP} minus $OD_{TP} > 0.5$). To further determine the true status of these eight samples, we used Western blotting to analyze anti-HEV IgM; the results showed that only the three sera with values for OD_{IP} minus OD_{TP} of >0.5 reacted with the dimer of polypeptide 459-607 and that the five others did not react with either of the polypeptides (Fig. 3), highly indicating that E4, E14, and

TABLE 3. Nonspecific binding of sera to ORF2 polypeptides

Serum sample	OD_{450} in IgG ELISA[a]		IgG ELISA result	OD_{450} in IgM ELISA[a]		IgM ELISA result	Genelab test result (OD_{450})	ALT level (U/liter)
	Polypeptide 459-607	Polypeptide 472-607		Polypeptide 459-607	Polypeptide 472-607			
E04	2.391	0.4	+	2.506	1.206	+	+ (2.376)	1,888
E14	2.395	0.581	+	1.949	1.375	+	+ (3.816)	296.6
E96	2.076	0.348	+	1.947	1.098	+	+ (3.580)	490.9
E08	1.003	0.365	+	0.819	0.504	−	+ (0.702)	17
E36	0.395	0.25	−	0.661	0.545	−	+ (0.753)	35
E51	0.569	0.357	−	0.508	0.525	−	+ (0.876)	12
E63	0.405	0.385	−	0.662	0.646	−	+ (0.645)	35
E90	0.553	0.493	−	0.822	0.614	−	+ (0.662)	26

[a] Microplate wells were coated with the immunodominant ORF2 polypeptide, covering amino acids 459 to 607, and the truncated polypeptide, covering amino acids 472 to 607.

E96 were truly positive and that the others were negative for anti-HEV IgM.

Additionally, we performed other experiments to gain more evidence to support the above results. Since specific IgM and IgG immune responses to HEV occur early in the acute phase of the infection, anti-HEV IgM and IgG may be detected simultaneously in the vast majority of acute infections (21). Thus, we tested anti-HEV IgG in these eight sera and found that all three IgM-positive sera were also positive for IgG, whereas only one of the five IgM-negative sera was positive for IgG (Table 3). Measurement of ALT levels in the eight sera showed that only E4, E14, and E96 were abnormal, while five others had no elevation (Table 3). Moreover, sera E14 and E96 had detectable HEV RNA, yet none of the five other sera were positive. Together, these results are supportive of the above findings that among the eight samples with high OD values, only the three sera with OD_{450} differences of >0.5 were truly positive, while the other five were negative for anti-HEV IgM, indicating that the truncated polypeptide 472-607 may be used to exclude false-positive results caused by nonspecific binding.

DISCUSSION

In the present study, we demonstrated that aa 459 to 607 in ORF2 of genotype 4 HEV form the immunodominant B-cell epitopes in natural infection and that the antigenicity of the immunodominant polypeptide depends upon dimerization. Based on the poor reactivity of anti-HEV to the truncated immunodominant polypeptide, we developed an indirect ELISA to exclude false-positive results in the detection of anti-HEV IgM.

The key factor in determining the reliability of an ELISA for measuring antibody is the coating antigen, which should cover the immunodominant B-cell epitopes. In our previous study, we identified a subregion of the HEV genotype 1 ORF2 capsid protein, spanning aa 459 to 607, as the minimal polypeptide to form the immunodominant epitopes, and an ELISA based on this immunodominant polypeptide was highly sensitive when human or nonhuman primate sera were tested (29). In the present study, we cloned ORF2 fragments of HEV genotype 4, which accounts for most hepatitis E cases in China, and prepared the corresponding polypeptides to test for anti-HEV IgM and IgG in naturally infected persons. Similar to previously reported results (29), both anti-HEV IgM and IgG reacted only with the immunodominant polypeptide, not with the truncated polypeptide, in the ELISA (Fig. 2 and Table 2). Thus, we proposed that if a serum reacted with the truncated polypeptide, the reactivity might be caused by nonspecific binding. To test our assumption, we screened 117 anti-IgM-positive (by Genelabs kit) serum samples with the ELISA developed in this study and found that eight sera reacted with both the immunodominant and truncated polypeptides (Table 3): three of them had considerably different OD_{450} values, and five other sera had comparable values. The results from further studies, including Western blot analyses (Fig. 3), simultaneous detection of anti-HEV IgG (Table 3), measurement of ALT levels (Table 3), and detection of HEV RNA, all support the conclusions that the three sera with significant differences between OD_{IP} and OD_{TP} were truly positive for anti-HEV IgM and that the five samples with comparable OD_{450} values were negative for anti-HEV IgM. Therefore, the truncated polypeptide may be used as a nonreactive element to exclude nonspecific binding in the detection of anti-HEV IgM by indirect ELISA.

The fact that the antigenicity of the immunodominant polypeptide depends upon its dimeric form should be the basis for the assay developed in this study. As demonstrated before (15) and shown in Fig. 2 in the present study, natural infection-induced anti-HEV reacted only with the dimer of the immunodominant polypeptide, not with the monomer. The truncated polypeptide did not form dimers (Fig. 2), leading to nonrecognition by anti-HEV. Thus, serum samples that react with the truncated polypeptide may contain nonspecifically binding immunoglobulin. Since the truncated polypeptide has identical residues to those in the immunodominant polypeptide, a sample that nonspecifically reacts with the truncated polypeptide may also nonspecifically bind to the immunodominant polypeptide, leading to a false-positive result.

It is notable that the event of a positive in the Genelabs test and a negative in the assay in the present study occurred with 70.9% of the serum samples (83 of 117 samples), while the results of retesting of 159 negative sera were in high agreement in both assays, indicating poor agreement for the IgM-positive results. We consider that these 83 discordant sera were mostly, if not all, false-positive results because of the following points: (i) all of these samples had OD_{450} values of <1 in the Genelabs test (data not shown), like the five false-positive results presented in Table 3; (ii) all sera had normal ALT levels; and (iii) none of the 43 sera analyzed by Western blotting reacted with the immunodominant polypeptide of ORF2.

One limitation of this study is that a small number of HEV RNA-positive sera were used in setting the criteria for specifically immunoreactive results; when a large number of samples are included, the cutoff may be changed. However, the present study provides several lines of evidence that the reactivity of a serum to the truncated immunodominant polypeptide is caused by nonspecific binding. By detection in parallel with ELISAs based on the ORF2 immunodominant and truncated polypeptides, false-positive results in the detection of anti-HEV IgM may be further eliminated. This would be valuable for diagnosing hepatitis E more accurately.

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