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Serotype-Specific Antigen ELISA for Detection of Chiba Virus in Stools

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Chiba virus (CV), a Norwalk-like virus (NLV), was first identified as a cause of oyster-associated outbreak of gastroenteritis that occurred in Chiba prefecture, Japan, in 1987. An enzyme-linked immunosorbent assay (ELISA), based on hyperimmune antisera to recombinant baculovirus-expressed capsid proteins of CV (rCV), was developed to detect CV antigen in stools. No cross-reactions were observed with other enteric viruses including enteroviruses, rotaviruses, astroviruses, or enteric adenoviruses. The ELISA was used to screen 101 stools collected from 16 oyster-associated outbreaks of acute gastroenteritis. Twelve stools (11.9%) from seven outbreaks were positive for CV antigen. Ten rCV ELISA-positive strains were confirmed by RT-PCR and nucleotide sequencing. ELISA-positive strains showed 96–100% nucleotide sequence identity to each other, though they were obtained nine years apart. Phylogenetic analysis demonstrated that all ten strains clustered with the prototype CV in genogroup I viruses. We concluded that the antigen ELISA described in this study is highly type-specific, and that this method should be useful for epidemiological surveys of Chiba virus infections. ***J. Med. Virol. 62:233–238, 2000.*** © 2000 Wiley-Liss, Inc.

KEY WORDS: Norwalk-like virus; recombinant protein; ELISA; virus-like particles

INTRODUCTION

Norwalk-like viruses (NLVs) are a genetically and antigenically diverse group of viruses that are classified as members of the family Caliciviridae [Cubitt et al., 1995; Kapikian et al., 1996; Estes et al., 1997; Pringle, 1998]. NLVs have been tentatively divided into two genogroups on the basis of sequence identities: Genogroup I is represented by Norwalk virus, Southampton virus, and Desert Shield virus; and Genogroup II by Snow Mountain agent, Mexico virus, Hawaii virus, and Bristol virus [Wang et al., 1994; Ando et al., 1995]. NLVs are often associated with outbreaks and sporadic cases of acute gastroenteritis in adults, school-age children, and infants in Europe, the Americas, and South Africa as well as Japan [Kaplan et al., 1982; Kobayashi et al., 1991; Norcott et al., 1994; Wolfaardt et al., 1995; Nakayama et al., 1996; Matsuno et al., 1997].

NLVs contain a 7.5–7.7 kb single-stranded, positive-sense RNA molecule that is polyadenylated at the 3′ end and presumably attached by a small protein called VPg at the 5′ end. Complete nucleotide sequences of NLVs demonstrated that the genome RNA has three open reading frames (ORFs) [Jiang et al., 1993; Lambden et al., 1993; Dingle et al., 1995]. The ORF1, the largest ORF, encodes a polyprotein including helicase, protease, and RNA-dependent RNA polymerase. The second ORF (ORF2) encodes the major viral capsid protein of 58 kDa. The third ORF (ORF3) is mapped at the 3′ end and codes for protein of unknown function.

Routine diagnosis of NLV infection has been achieved by observing virus particles in stool with electron microscopy (EM) due to the lack of a cell culture system for NLVs. Although EM is useful as a screening method, EM is time-consuming and relatively insensitive due to poor shedding of NLVs into stool [Haruki et al., 1991]. Recent progress on molecular cloning of NLVs' genome led to the development of reverse transcription-polymerase chain reaction (RT-PCR) assays for the diagnosis of NLV infection [De Leon et al., 1992; Jiang et al., 1992a]. Many of the RT-PCR assays, however, were not so efficient, owing to the considerable genomic diversity among NLVs [Moe et al., 1994; Norcott et al., 1994; Wang et al., 1994].

When the ORF2 gene was expressed by recombinant baculovirus system, the expressed capsid proteins formed empty virus-like particles (VLPs). To date, the production of six genetically distinct VLPs has been reported [Jiang et al., 1992b, 1995b; Dingle et al., 1995;

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Leite et al., 1996; Green et al., 1997; Hale et al., 1999]. Baculovirus-expressed VLPs of NV, MX, and Hawaii virus were used for seroepidemiological studies by an enzyme-linked immunosorbent assay (ELISA) [Green et al., 1993; Numata et al., 1994; Parker et al., 1994]. Antigen ELISAs for the detection of the viral antigen in stools were also developed by using antisera to a recombinant capsid proteins [Graham et al., 1994; Jiang et al., 1995a].

The present study describes the development of an antigen ELISA based on hyperimmune sera to baculovirus-expressed capsid protein of Chiba virus (CV), a Japanese strain in genogroup I [Kasuga et al., 1990; Utagawa et al., 1994]. To evaluate the specificity of the ELISA, we determined the nucleotide sequences of ELISA-positive strains and phylogenetically analyzed their genotype.

MATERIALS AND METHODS

Stools and Electron Microscopic Examination

Stools were obtained from 118 patients involved in 16 oyster-associated outbreaks of nonbacterial acute gastroenteritis that occurred during 1986–96 in Aichi prefecture, Japan. A 10% stool suspension was prepared with 10 mM phosphate buffered saline (PBS), pH 7.4 and clarified by centrifugation at 10,000 × *g* for 20 min. The supernatants were stored at 4°C or frozen at −20°C until use. For EM, the supernatants were further centrifuged at 100,000 × *g* for 150 min in a Hitachi RPS-40T rotor and the pellets were resuspended in a few drops of distilled water. NLVs were negatively stained with 3% phosphotungstic acid (pH 7.0) and examined with a JEOL JEM-100CX electron microscope.

Expression of Recombinant CV Capsids (rCV)

NLV used for the expression of the capsid protein was the prototype Chiba virus, NLV/Chiba407/1987/JP strain, that was derived from a stool collected during an oyster-associated outbreak of gastroenteritis in Chiba prefecture, Japan, in 1987 [Kasuga et al., 1990]. Purification of the virion, preparation of the RNA and cDNA synthesis were performed as described previously [Utagawa et al., 1994]. Amplification of a 1.6 kb fragment containing an entire ORF2 was done by using a forward primer G1F1 (5′-TGCCCGAATTCGTAAATGAT-3′) and a reverse primer CV-U4 (5′-GCGAATTCTTATCTACGGACACCAAGCCTAC-3′). The amplified fragment was inserted into the pCR 2.1 vector (TA cloning system, Invitrogen). After *Eco*RI digestion of the plasmid, the digested fragment containing ORF2 gene was ligated into baculovirus transfer vector pVL1392. The orientation of the insert was confirmed by digestion with restriction enzymes. The transfer vector was cotransfected with linearized wild-type *Autographa Californica* nuclear polyhedrosis virus DNA (BaculoGold, PharMingen) to transform Sf9 cells by lipofectin-mediated method to generate recombinant baculoviruses according to the manufacturer's procedure. The recombinant baculovirus was selected by three rounds of plaque purification and amplified once to prepare a stock virus. To express the capsid protein of Chiba virus, Tn5 cells, an insect cell line derived from *Trichoplusiani,* BTL-Tn 5B1-4 (Invitrogen, San Diego, CA) were infected with the recombinant viruses at a multiplicity of infection 1–3 and incubated in Excell 405 medium (JRH Biosciences, Lenexa, KS) at 26.5°C for 5–6 days. Ten μM Leupeptin (Sigma Chemicals, St. Louis, MO) was added to the medium at 3 days after infection to reduce the formation of the smaller particles. When the incubation was completed, virus-like particles (VLPs) released into the supernatant were purified in CsCl equilibrium density gradient ultracentrifugation followed by sucrose density gradient centrifugation [Jiang et al., 1995b].

Immunization of Laboratory Animals

Hyperimmune sera to rCV were obtained from guinea pigs and rabbits. The animals were immunized by one subcutaneous injection of the purified VLPs, 250 μg per guinea pig, and 500 μg per rabbit, in Freund's complete adjuvant. After one month, the animals received 2 or 3 booster injections of a half dose in Freund's incomplete adjuvant at intervals of one week. The animals were bled one week after the last booster injection.

ELISA for Detection of CV Antigen

Duplicate wells of 96-well microtiter plates (Maxisorp, Nunc, Denmark) were coated with 100 μl of 1:10,000 diluted pre- or post-immunization rabbit serum in a coating buffer (0.1 M carbonate-bicarbonate buffer, pH 9.6) overnight at 4°C. The wells were washed twice with phosphate-buffered saline containing Tween 20 (PBS-T), and then blocked with 2% bovine serum albumin in PBS (1% BSA-PBS). A 100 μl of stool sample (10% suspension in 1% BSA-PBS) was added to wells and incubated overnight at 4°C. After washing the wells 4 times with PBS-T, 100 μl of 1:5,000 diluted hyperimmune serum from guinea pig with 1% BSA-PBS was added to the wells and the plate was incubated for 1 hr at 37°C. The plate was washed 4 times with PBS-T, and then horseradish peroxidase-conjugated rabbit anti-guinea pig antibody (Zymed, 1:10,000 in 1% BSA-PBS) was added to each well. After incubation for 1 hr at 37°C, the plate was washed 4 times with PBS-T and 100 μl of substrate *o*-phenylenediamine and H_2O_2 was added. The plate was left for 10 min at room temperature and then the reaction was stopped with 50 μl of 2 M H_2SO_4. Absorbance at 492 nm (A_{492}) of the wells coated with postimmune antiserum (P) or preimmune serum (N) was measured with a microplate reader (Corona MTP-32).

Detection of NLVs in Stool by RT-PCR

Extractions of viral RNA from the stools and cDNA synthesis were performed as described above. A primer pair G1F1 and G1R1 (5′-CCAACCSARCCATTRTACATTTG-3′) was designed based on the conserved capsid genes of the published NLVs. The primer pair G1F1 and G1R1 was expected to produce fragments of

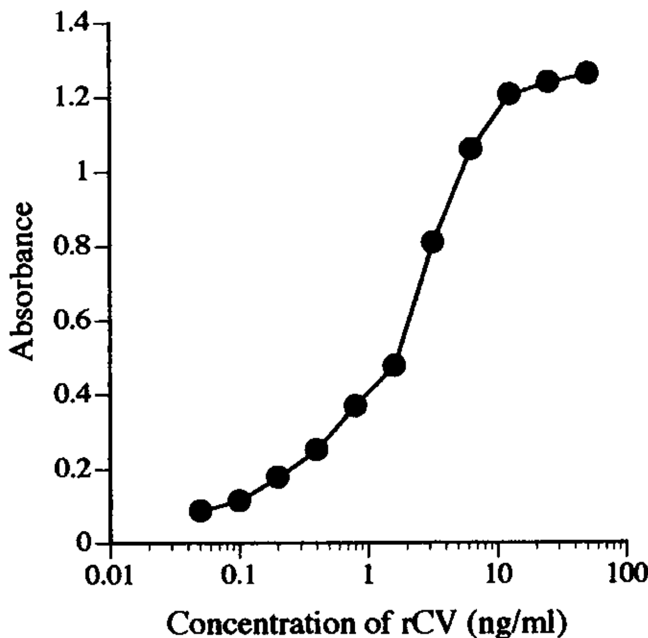

Fig. 1. Sensitivity of the antigen ELISA for the detection of Chiba virus. Two-fold serial diluted VLPs of rCV (100 μl/well) were added to each well.

319 bp. The reaction mixture contained 1.25 U Taq polymerase (Behringer), 5 μl of 10 × PCR buffer (100 mM Tris-HCl, 15 mM $MgCl_2$, 500 mM KCl, pH 8.3), 4 μl of 25 mM deoxynucleotide mixture, 0.5 μl of 50 μM each primer, 2 μl of cDNA, and sterile distilled water in the total 50 μl. After an initial denaturation at 94°C for 5 min, 30 cycles of amplification were carried out. Each cycle consisted of the denaturation for 1 min at 94°C, the annealing for 1 min at 55°C, and the extension for 2 min at 72°C, that was followed by a final incubation for 7 min at 72°C.

Nucleotide Sequence Analysis

The RT-PCR-amplified products were cloned into pGEM T cloning vector (Promega, Madison, Wisconsin) and the insert was sequenced by using SequiTherm Long-Read Cycle Sequencing Kit (Epicentre Technologies, Madison, Wisconsin) and Model 400 Automated DNA Sequencer (LI-COR Inc., Lincoln, NE). Multiple sequence alignment was carried out using the GENETYX package (Software Development Co., Ltd., Tokyo) and a dendrogram was generated by UPGMA.

The nucleotide sequence data reported in this paper will appear in the DDBJ, EMBL, and GenBank nucleotide sequence databases, with accession numbers from AB021987 to AB021996.

RESULTS AND DISCUSSION

The antibody titers of the hyperimmune rabbit and guinea-pig sera against rCV were 1:3,200,000 and 1: 820,000, respectively, when hyperimmune sera were tested by indirect ELISA [Graham et al., 1994]. A sandwich-type antigen-detection ELISA was found to be optimal when the rabbit antiserum was used as capture antibody and the guinea pig antiserum as detector antibody. The specificity of ELISA for rCV antigen was evaluated by testing the representative human enteritis viruses including coxsackievirus of types A2, A9, B2 and B6, echo virus types 9 and 30, enteric adenovirus types 40 and 41, rotavirus serotypes 1 to 4, and astrovirus serotypes 1 and 2. None of these viruses gave a positive reaction by antigen ELISA for CV. The antigen ELISA strongly reacted with homologous VLPs of rCV, but two heterogous recombinant VLPs derived from a genogroup I NLV (NLV/Aichi124/1989/JP) and genogroup II NLV (NLV/Aichi1876/1996/JP) gave no signal. The former is genetically very close to the prototype Norwalk virus (NLV/Norwalk/1968/US), and the latter is genetically similar to Hawaii virus (NLV/Hawaii/1971/US) (data not shown and in preparation). A panel of 100 stools from healthy children showed a mean A_{492} (P–N) of 0.026 with a standard deviation (SD) of 0.025 and a mean P/N ratio of 1.32 with a SD of 0.19. Samples were considered positive if the A_{492} (P–N) was >0.10 (mean + 3 SD) and P/N ratio was >1.89 (mean + 3 SD). The sensitivity of the ELISA was determined by using serially diluted rCV with 1% BSA-PBS (Fig. 1). The assay was capable of detecting as little as 0.01 ng of rCV antigen, that is equivalent to 6.3×10^5 VLPs supposing one VLP is formed by 180 capsid subunits with a molecular weight of 58 kDa [White et al., 1997].

TABLE I. Detection of NLVs in Stools From Oyster-Associated Outbreaks

Outbreak	Year	EM (No. positive/tested)	ELISA (No. positive/tested)
1	1986	0/16	2/11
2	1987	0/12	0/5
3	1987	0/7	0/3
4	1988	1/5	1/5
5	1988	1/3	0/2
6	1989	4/11	0/8
7	1989	1/4	0/2
8	1990	0/6	1/6
9	1990	1/6	0/6
10	1991	4/7	0/7
11	1993	3/14	0/14
12	1994	5/6	5/6
13	1995	3/6	1/6
14	1996	6/8	0/8
15	1996	NT[a]	1/5
16	1996	4/7	1/7
Total		33/118 (28.0%)	12/101 (11.9%)

[a]Not tested due to insufficient amount of samples.

Stools from 118 gastroenteritis patients involved in 16 oyster-associated outbreaks were tested by EM and antigen ELISA. As shown in Table I, NLVs were detected in 33 (28.0%) of the 118 stools from 11 of 15 outbreaks by EM, whereas 12 (11.9%) of 101 stools from 7 of 16 outbreaks were positive by ELISA. This result does not mean that the ELISA is less sensitive than EM. EM does not consider the serotype but ELSA is highly serotype-specific. It is known that the stools derived from oyster-associated outbreaks contain miscellaneous genetic types of NLVs [Sugieda et al., 1996].

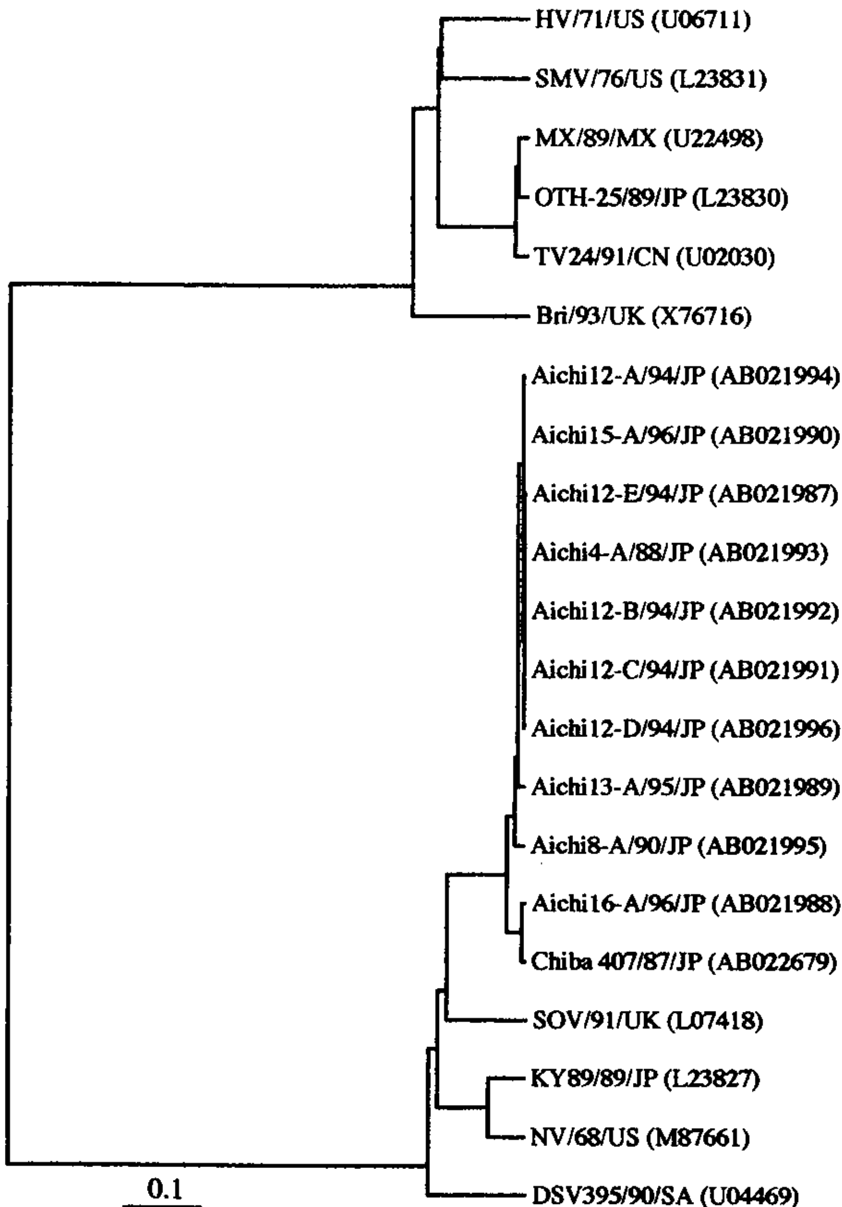

Fig. 2. Determination of the genotype of the ELISA-positive strains. The nucleotide sequences were analyzed by the GENETYX package. Tree topology was inferred by the UPGMA cluster analysis method. Numbers indicate the outbreak cases indicated in Table I. A–E denote different stools derived from the same outbreak. The length of each horizontal line is proportional to the genetic distance. GenBank sequence accession numbers of NLVs are given in parentheses. Chiba 407/87/JP is the prototype strain of Chiba virus.

It should be noted that the assay was capable of detecting the antigen in EM-negative stools as seen in the outbreaks in 1986 and 1990 (Table I), suggesting that hyperimmune sera could capture the soluble or degraded capsid protein in the stools.

To identify the genotype of ELISA-positive strains, partial capsid genes (314 bases in 5′ end of ORF2) of each of 10 strains from five different outbreaks were sequenced. The 10 strains shared 95–100% nucleotide identity and 96–100% amino acid identity, indicating that these strains were genetically closely related to each other even though they were obtained 9 years apart. The ELISA-positive 10 strains had the highest nucleotide identity with CV (95–96%), followed by Southampton virus (SV) (80–81%), NV (80–81%), and Desert Shield virus (DSV) (76–77%), when compared to GI NLVs. The nucleotide identity to genogroup II viruses including Snow Mountain agent, Hawaii virus, Mexico virus, Toronto virus and Bristol virus were as low as 62–67%. The clustering by the dendrogram was depicted in Figure 2. The 10 ELISA-positive strains formed one group with the prototype of Chiba virus in genogroup I.

Antigenic classification of NLVs has been mainly performed by immune electron microscopy (IEM) using stool specimens and a set of acute and convalescent sera from patients with acute gastroenteritis. Okada et al. [1990] grouped NLVs from sporadic infections and outbreaks into nine antigenic types (SRSV1 to SRSV9). A similar study in the United Kingdom showed that NLVs from 10 cases were grouped into four antigenic types (UK1 to UK4) [Lewis, 1991] and six antigenic types were reported in the United States [Lewis et al., 1995]. Further studies, however, have been hampered

due to the inability of NLVs to grow in cell cultures and limited volumes of the paired sera. In addition, the specificity of patients' sera is uncertain, because the historical background of the infection, especially the past infection, is unknown.

In this study, we developed antigen ELISA for the detection of CV utilizing hyperimmune antisera to baculovirus-expressed capsid protein. This antigen ELISA was highly specific for Chiba virus, with no reactions to other enteric viruses and heterologous VLPs. When the homologous VLPs of rCV were used, the ELISA detected 0.01 ng of protein. This corresponds to approximately 6.3×10^5 virions and is equivalent in sensitivity to that of antigen ELISA for NV and MX [Jiang et al., 1995a,c]. We used the antigen ELISA to clarify the prevalence of Chiba virus in the outbreaks of gastroenteritis, and 7 (43.8%) of 16 outbreaks were found to be associated with CV. Therefore, Chiba virus proved to be one of the causative NLV strains of oyster-associated gastroenteritis in Aichi prefecture during 1986–96. Some of the ELISA-positive samples were genetically characterized by RT-PCR and subsequent nucleotide sequencing. Phylogenetic analysis of the capsid region showed that ELISA-positive strains clustered with the prototype Chiba virus. These results indicated that NLVs detected by our ELISA are antigenically and genetically similar to the prototype Chiba virus. Based on the sequences of the RNA polymerase gene, NLVs have been classified into two major genogroups, Norwalk-like or genogroup I virus and Snow Mountain-like or genogroup II virus [Wang et al., 1994]. Two major genogroups are antigenically distinct from each other, and a good relationship is observed between these genogroups and antigenic groups [Jiang et al., 1995a]. Recent detailed phylogenetic analysis, however, demonstrated that each genogroup can be further divided into subgenogroups [Ando et al., 1995; Hardy et al., 1997]. As the antigenic diversity within the NV genogroup remains unclear, the newly developed ELISA should be a valuable tool for antigenic typing of NV genogroup strains. In our results, EM gave a higher detection rate than ELISA. This is probably because EM does not take serotype into consideration. Jiang et al. [1995a] indicated that the antigen ELISA for rNV and rMXV are quite specific for the prototype NV and MXV strains, respectively. Some of the ELISA-negative outbreaks may be associated with other serotypes of NLVs. Further success in expression of antigenically different NLVs is needed to develop the antigenic classification system for NLVs. ELISA is simple and rapid, and requires only small amounts of stools. As ELISA is capable of efficiently processing many samples, this method should be useful for the large-scale epidemiological studies of Chiba virus infection.

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Expression of Recombinant Capsid Proteins of Chitta Virus, a Genogroup II Norwalk Virus, and Development of an ELISA to Detect the Viral Antigen

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Abstract: **The second open reading frame (ORF2) gene of the Chitta virus (CHV) was cloned to construct a recombinant baculovirus. The CHV ORF2 is predicted to encode a capsid protein of 535 amino acids (aa). CHV showed a high aa identity in the capsid region with genogroup II Norwalk virus (NV) (65–85%), but a low aa identity with genogroup I NV (44–46%). Phylogenetic analysis of the ORF2 gene demonstrated that CHV is genetically closely related to the Hawaii virus included in genogroup II NV. The recombinant capsid protein of CHV (rCHV) self-assembled to form empty virus-like particles (VLPs) when expressed in insect cells with the recombinant baculovirus. An enzyme-linked immunosorbent assay (ELISA) based on antisera to rCHV was developed to detect CHV antigen in stools. The antigen ELISA appeared to be highly specific to both rCHV and CHV-like strains. In addition, combined use of antigen ELISAs using antibodies against two antigenically distinct recombinant VLPs, the recombinant Chiba virus (rCV) and recombinant Seto virus (rSEV), enabled us to determine the genetic as well as antigenic relationship among these three viruses.**

Key words: **Norwalk-like virus, Recombinant protein, Virus-like particles, ELISA**

Norwalk virus (NV), a single member of the genus Norwalk-like viruses in the family *Caliciviridae*, is a major cause of sporadic cases or outbreaks of acute gastroenteritis in Japan and worldwide (13, 14, 18, 19, 29). Virological analyses of NV have been hindered by the inability to grow the virus in cell cultures and the lack of animal models. However, recent progress in the molecular cloning and sequence analysis of the NV genome enabled us to genetically divide NV into two genogroups; genogroup I (GI), including the Norwalk/68, Southampton and Desert Shield viruses, and genogroup II (GII), including the Snow Mountain, Mexico and Hawaii viruses (17, 27).

NV contains a single-stranded positive-sense RNA genome of approximately 7.5 kb that encodes three potential open reading frames (ORFs) (12, 15). The RNA genome is polyadenylated at the 3′ end, and a small protein called VPg is attached at the 5′ end. The ORF1 located in the 5′ end half of the genome is predicted to encode a polyprotein precursor of nonstructural proteins including helicase, protease, and polymerase. The second ORF (ORF2) encodes a capsid protein of approximately 58 kDa and partially overlaps with ORF1. The third ORF (ORF3) is mapped between ORF2 and the 3′ end of the genome, and encodes a small protein of unknown function. When the entire ORF2 or a region spanning ORF2 to the poly (A) tract including ORF3 was expressed by a recombinant baculovirus, the capsid pro-

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Abbreviations: aa, amino acid; CHV, Chitta virus; CV, Chiba virus; ELISA, enzyme-linked immunosorbent assay; EM, electron microscope; HV, Hawaii virus; IEM, immune electron microscopy; kDa, kilodalton; MW, molecular weight; NV, Norwalk virus; ORF, open reading frame; PBS, phosphate-buffered saline; RT-PCR, reverse transcription-polymerase chain reaction; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SEV, Seto virus; VLPs, virus-like particles.

teins thus expressed spontaneously self-assembled into empty virus-like particles (VLPs) (2, 9, 11). These VLPs have been successfully used as antigen in the detection of antibodies to NV (5, 24). Seroepidemiological surveys have shown that NV is a common agent in hosts of almost all ages, and that children usually acquire antibodies to NV at an early age in both developed and developing countries (4, 22, 23). Although antigen ELISAs using antisera to VLPs have been developed for the detection of virus antigens in stools (3, 8), the detection rate is unexpectedly low due to the high specificity of the assay and the large antigenic diversity among NV (10, 20). Expression of VLPs of all serotypes of NV is necessary to improve the efficiency of the assay.

This paper describes the cloning of the capsid protein gene of the Chitta virus (CHV), a GII NV strain isolated in Aichi Prefecture, Japan, in 1996, and the subsequent expression of the recombinant capsid protein (rCHV) by using a recombinant baculovirus. We also developed an antigen ELISA based on antisera to rCHV for the detection of CHV-like strains in stools.

Materials and Methods

Stools. A total of 15 stool samples from three food-borne outbreaks of acute gastroenteritis that occurred in Aichi Prefecture, Japan, were used. Two outbreaks in 1989 and 1992 were associated with meals served at a hotel, and the third, which occurred in 1996, was an oyster-associated outbreak.

Cloning and construction of baculovirus recombinants. A stool sample (1876/96 in Table 2) collected from an outbreak of gastroenteritis in Aichi Prefecture in 1996 was used as a source of viral RNA for cDNA synthesis. The NV was designated as Chitta virus (CHV) based on the location of the outbreak. Electron microscopic observation indicated that the CHV virion was approximately 35 nm in diameter and the surface structure was characterized as a typical small round-structured virus. The viral RNA was extracted from 100 μl of the 10% stool suspension using Trizol™ (Gibco BRL, Gaithersburg, Md., U.S.A.) and finally dissolved in 20 μl of distilled water. Complementary DNA was synthesized using oligo $(dT)_{15}$ (Promega Co., Madison, Wisc., U.S.A.) and a Moloney murine leukemia virus reverse transcriptase (Gibco BRL) as previously described (6). The entire ORF2 was amplified by semi-nested PCR using the first forward primer G2F1 (5′-TGGGAGGGCGATCGCAATCT-3′) and the reverse primer G2R0 (5′-CCATTACTGAACCCTTCTACGCC-3′), and the second forward primer G2F2 (5′-GTGAATGAAGATGGCGTCGA-3′) and the reverse primer G2R0. The reaction mixture contained 2.5 units of Takara Ex Taq (Takara Shuzo Co., Ltd., Kyoto, Japan), 10 μl of 10×PCR buffer, 8 μl of 25 mM dNTPs, 1 μl of 50 μM each primer, 5 μl of cDNA, and sterile distilled water to make a total of 100 μl. After an initial denaturation at 94 C for 5 min, 35 cycles of amplification were performed using the GeneAmp PCR System 9600 (PE Applied Biosystems, Foster City, Calif., U.S.A.). Each cycle consisted of denaturation at 94 C for 1 min, primer annealing at 55 C for 1 min, and extension reaction at 72 C for 2 min followed by final extension at 72 C for 7 min.

An approximately 1.6 kb PCR product was cloned into a TA cloning vector, pCR2.1 (Invitrogen, San Diego, Calif., U.S.A.), to generate pCR[CHV]. The insert was sequenced by using a SequiTherm Long-Read Cycle Sequencing Kit (Epicentre Technologies) and a Model 400 Automated DNA Sequencer (LI-COR Inc., Lincoln, Neb., U.S.A.). After *Eco*RI digestion of plasmid pCR[CHV], the fragment containing the entire ORF2 gene was ligated with a baculovirus transfer vector, pVL1392 (Pharmingen, San Diego, Calif., U.S.A.), at the *Eco*RI site to produce pVL[CHV]. Sf9 cells (Riken Cell Bank, Tsukuba, Japan) derived from insect *Spodoptera frugiperda* were cotransfected with 50 ng linearized wild-type *Autographa californica* nuclear polyhedrosis virus DNA (BaculoGold Kit, Pharmingen) and 1 μg pVL[CHV] by the lipofectin-mediated method as specified by the manufacturer. Recombinant viruses, designated as Ac[CHV], were selected by two rounds of plaque purification (25), propagated once in Sf9 cells, and stored as seed viruses.

Expression and purification of the recombinant protein. Tn5 cells derived from *Trichoplusiani*, BTL-Tn 5B1-4 (Tn5) (Invitrogen), were used for large-scale expression (28). Tn5 cells were infected with the recombinant baculovirus Ac[CHV] at a multiplicity of infection of 10, and incubated in EX-cell 405 medium (JRH Biosciences, Lenexa, Kan., U.S.A.) at 26.5 C for 5–6 days. Expression of protein was monitored by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by staining with Coomassie brilliant blue. The culture medium was clarified by centrifugation at $10{,}000\times g$ for 30 min, then the VLPs in the supernatant were concentrated by centrifugation at $100{,}000\times g$ for 2 hr. The pellet was resuspended in a few drops of distilled water and examined with an electron microscope (EM) (JEOL JEM-100CX). The VLPs were further purified by CsCl equilibrium gradient and sucrose density gradient centrifugation (9). The purified VLPs were used for the immunization of animals.

Preparation of antiserum. Hyperimmune sera to recombinant capsid protein of CHV (rCHV) were prepared in rabbits by subcutaneous injection of the purified

500 μg VLPs in Freund's complete adjuvant. After one month, the animals received two or three booster injections of 250 μg of the VLPs in Freund's incomplete adjuvant at intervals of one week. The animals were bled one week after the last booster injection.

ELISA. Duplicate wells of microtiter plates (Maxisorp, Nunc) were coated with 100 μl of 1:10,000 diluted pre- or post-immunization rabbit serum in coating buffer (0.1 M carbonate-bicarbonate, pH 9.6) overnight at 4 C. The wells were washed twice with phosphate-buffered saline containing Tween 20 (PBS-T), and then blocked with 2% bovine serum albumin in PBS (1% BSA-PBS). One hundred microliters of 10% stool suspension in 1% BSA-PBS was added to the wells and incubated for 1 hr at room temperature. After washing the wells four times with PBS-T, 100 μl of horseradish peroxidase-conjugated rabbit hyperimmune serum to rCHV was added to each well. After incubation for 1 hr at room temperature, the plate was washed four times with PBS-T and 100 μl of substrate *o*-phenylenediamine was added. The plate was left for 10 min at room temperature, and then the reaction was stopped with 50 μl of 2 M H_2SO_4. Absorbance at 492 nm (A_{492}) was measured with a microplate reader (Corona MTP-32). A P/N ratio of >2.0 and a $P-N$ of >0.15 were considered to be positive reactions, where P is the absorbance of the well coated with hyperimmune serum and N is the absorbance of the well coated with preimmune serum.

Detection of NV in stool by RT-PCR. Extraction of viral RNA from the stool, cDNA synthesis, and PCR were performed as described above. Two sets of primer pairs, G1F1 (5'-TGCCCGAATTCGTAAATGAT-3') and G1R1 (5'-CCAACCSARCCATTRTACATTTG-3'), and G2F1 and G2R1 (5'-GCATAACCATTRTACATTCT-3'), were designed based on the nucleotide sequence of the capsid protein of GI and GII NV, respectively. The primer pairs G1F2 (5'-AATGATGATGGCGTCTAAGGA-3') and G1R1 for GI NV, and G2F2 (5'-TTGTGAATGAAGATGGCGTCGA-3') and G2R1 for GII NV were used in the nested PCR.

Nucleotide sequence analysis. The nucleotide sequences were analyzed using a SINCA package (Fujitsu, Ltd., Tokyo). Multiple substitutions were corrected by Kimura's two-parameter method. The dendrogram was inferred by the neighbor-joining method with the bootstrap option. The numbers at the branching points are the 50% threshold majority consensus value for 100 bootstrap replicates. The nucleotide sequences used in the analyses were Norwalk virus/68 (NV/68) (Accession number M87661), KY89 (L23828), OTH25 (L23830), Southampton virus (SOV) (L07418), Desert Shield virus (DSV) (U04469), Lordsdale virus (LV) (X86557), Bristol virus (BV) (X76716), Camberwell virus (CWV) (U46500), Toronto virus (TV) (U02030), Mexico virus (MXV) (U22498), Snow Mountain virus (SMV) (U70059), Melksham virus (MSV) (X81879), Alkland virus (AV) (U46039), Hawaii virus (HV) (U07611), Chiba virus (CV) (AB022679), and Seto virus (SEV) (AB031013).

The nucleotide sequence data of CHV will appear in the DDBJ/EMBL/GenBank nucleotide sequence databases with the accession number AB032758.

Results

Sequence Analysis of CHV

The nucleotide sequence analysis of pCR[CHV] indicated that the ORF2 of CHV is predicted to consist of 1,608 nucleotides (535 aa). Comparison of the ORF2 sequence with those of published NV strains revealed that CHV has 60–76% nucleotide (65–85% aa) identity with GII NV, whereas lower sequence identity to the strains of

Table 1. Percentage identities in pairwise comparisons of ORF2 nucleotide sequences (upper right) and deduced amino acid sequences (lower left) of NV strains

	CHV	HV	MSV	SMV	MXV	TV	OTH	LV	NV/68	KY89	SEV	SOV	CV	DSV
CHV	*	76	71	71	61	60	60	65	53	53	53	53	54	55
HV	85	*	70	70	60	60	61	65	53	53	52	53	54	56
MSV	76	75	*	94	65	65	65	63	52	52	52	54	54	55
SMV	76	75	98	*	66	66	65	64	53	51	52	54	55	55
MXV	70	72	68	68	*	97	97	65	52	52	52	53	52	54
TV	71	72	68	68	97	*	96	66	53	52	52	53	52	53
OTH	70	71	67	67	96	97	*	65	51	52	51	53	52	53
LV	65	65	63	63	66	67	66	*	46	52	51	53	53	54
NV/68	46	47	47	47	45	46	45	44	*	87	87	62	64	62
KY89	45	47	45	45	44	44	43	44	96	*	97	62	63	62
SEV	45	47	45	46	47	46	45	44	98	97	*	62	66	62
SOV	45	45	44	44	42	42	42	43	69	68	69	*	70	66
CV	45	46	45	45	42	43	43	44	72	71	71	75	*	65
DSV	44	47	46	47	45	45	44	44	68	67	68	67	66	*

GI NV (53–55% of nucleotides and 44–46% of aa) was observed (Table 1). A dendrogram based on the nucleotide sequence of the capsid gene demonstrated that CHV belongs to GII NV, and that CHV is genetically most closely related to the Hawaii virus (Fig. 1).

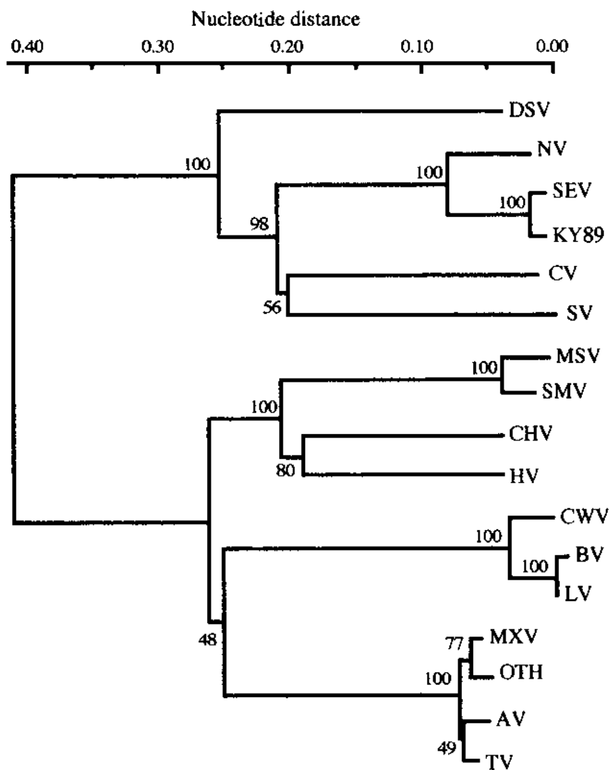


Fig. 1. Phylogenetic analysis of NV strains. The nucleotide sequences of the entire capsid protein genome, corresponding to nucleotides 5358 to 6950 of Norwalk virus/68 genome, were analyzed by the neighbor-joining method.

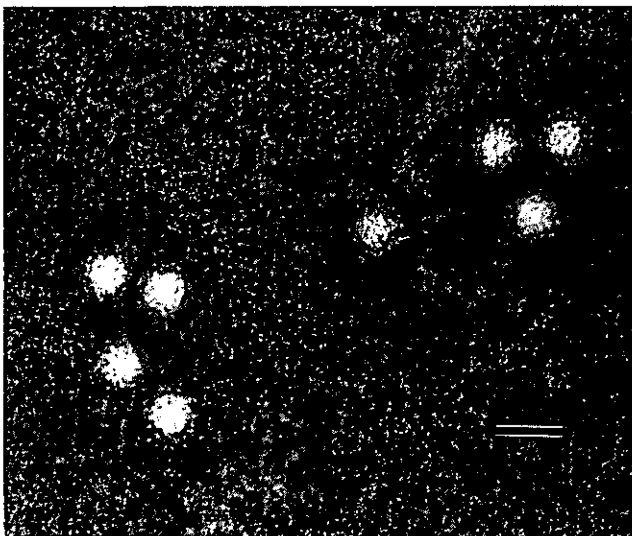
Expression of CHV Capsid Proteins in Insect Cells

The CHV ORF2 region was expressed using recombinant baculovirus Ac[CHV] in Tn5 cells. SDS-PAGE of expressed proteins in the culture medium and cell lysate at 3 days postinfection demonstrated a major protein band with a molecular mass of about 58 kDa (data not shown). The size of the protein was in agreement with the MW calculated from the 535 amino acids of the CHV ORF2 (MW 58,501). The release of the expressed protein into the medium reached a maximum at 6 days postinfection. The culture medium was ultracentrifuged and the pellet was examined by EM. Many round, empty virus-like particles (VLPs) with a diameter of 35 nm were observed. These rCHV particles were morphologically similar to the native CHV purified from the stool sample of the patient (Fig. 2). The typical yield of the purified VLPs was 0.1–0.2 mg per 2×10^7 insect cells.

Development of Antigen ELISA to Detect NV in Stool

Hyperimmune sera to rCHV VLPs were prepared by immunizing rabbits with purified CHV VLPs, and the sera were used in the development of antigen ELISA for the detection of CHV in stools. Antigen ELISAs to detect Chiba virus (CV) (submitted for publication) and Seto virus (SEV) (submitted for publication), both of which are included in GI NV, were performed in parallel to evaluate their respective specificity (Table 2). In the control experiments using 2 ng/ml of rCHV VLPs, the ELISA for the detection of rCHV specifically reacted

(A)



(B)

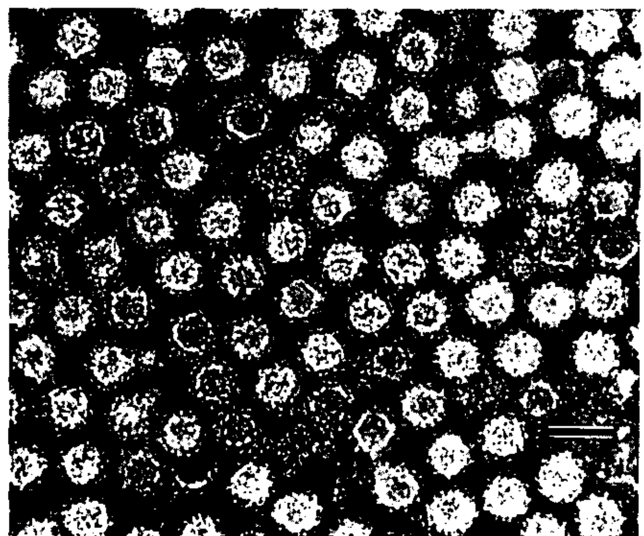


Fig. 2. Electron micrographs of the native virions of CHV purified from stool (A) and recombinant VLPs (B). The scale bars indicate 50 nm.

Table 2. Detection of NV strains by ELISA and RT-PCR

Case	Samples	Antigen ELISA for[a]			RT-PCR
		CHV	CV	SEV	
	rCHV[b]	**1.10**	0.007	0.015	N.T.[c]
	rCV[b]	0.008	**0.93**	0.024	N.T.
	rSEV[b]	0.010	0.018	**1.05**	N.T.
1	121/89	0.008	0.012	**1.31**	+
	122/89	0.001	0.000	**1.61**	+
	124/89	0.000	0.000	**0.525**	+
	125/89	0.000	0.000	**0.233**	−
	131/89	0.003	0.006	**1.48**	+
	132/89	0.000	0.004	**1.74**	+
	133/89	0.009	0.000	**1.54**	+
2	5/94	0.075	**1.73**	0.097	+
	6/94	0.034	**1.55**	0.058	+
	8/94	0.035	**1.66**	0.101	+
	9/94	0.043	**0.813**	0.038	+
	10/94	0.031	**1.55**	0.067	+
3	1874/96	**0.848**	0.000	0.000	−
	1875/96	**0.271**	0.000	0.000	−
	1876/96[d]	**1.60**	0.000	0.002	+

[a] Absorbance at 492 nm. Positive reactions are indicated in boldface.

[b] Purified VLPs were used at a concetration of 2 ng/ml, which is equivalent to 1.2×10^8 VLPs.

[c] Not tested.

[d] Stool was used for the expression of rCHV VLPs.

with the homologous antigen, but not with the heterologous rCV and rSEV antigens. The same result was obtained by the ELISA for the detection of rCV and rSEV. A total of 15 stools from three outbreaks of acute gastroenteritis were tested by the ELISA. The nucleotide sequence was determined in parallel using the RT-PCR products to identify the genotype of the strains (Table 2). In the outbreak that occurred in 1989 in a junior high school during an off-campus excursion, 179 of the 536 (33%) students developed acute gastroenteritis. A meal taken at a hotel during the trip was suspected to include the relevant food. Seven stool samples were positive for SEV antigen by ELISA, though one sample was negative for NV by RT-PCR. In the outbreak in 1994, 8 of 12 (67%) people became ill after eating raw oysters. Viral particles were observed in five stool samples by EM. All five samples were positive for CV by ELISA and also positive by RT-PCR. In the outbreak that occurred in 1996, illness was observed in 11 of 28 (39%) people who stayed in one hotel. A meal served in the hotel was suspected to be the cause. Of three stool samples, one was positive for viral particles by EM. All three samples were positive by the ELISA for CHV, though only one sample was positive by RT-PCR.

Overall, antigen ELISA efficiently detected antigenically homotypic strains but did not detect heterotypic strains. Sequencing analysis of RT-PCR product indicated that the 122/89 strain showed high nucleotide (97%) and aa (95%) identity with the prototype SEV. Similarly, the nucleotide sequence from the five CV-antigen ELISA-positive samples showed 95–96% identity with that of the prototype CV (15). These results indicated that antigen ELISA is highly specific to genetically, closely related strains.

Discussion

Antigenic classification of NV strains has been mainly performed by immune electron microscopy (IEM) using stool samples and a set of acute and convalescent sera from patients with acute gastroenteritis due to the inability of NV to propagate in cell cultures (16, 21). However, such studies have been restricted by a limited amount of clinical samples and the lack of standardized reagents. In the present study, we obtained the CHV capsid protein by using a recombinant baculovirus expression system. The expressed recombinant protein spontaneously assembled into virus-like particles (VLPs) morphologically similar to the native virions. These VLPs retained their immunogenicity in animals and the antiserum was used for the development of antigen ELISA. The ELISA reacted with the homologous serotype of VLPs only; it did not react with the heterologous serogroup of VLPs. When the serial dilution of VLPs was tested by the ELISA, as little as 0.01 ng of protein was detected (data not shown). This amount corresponds to approximately 6.3×10^5 virions, and in terms of sensitivity is equivalent to those of rNV and rMX antigen ELISAs (3, 8). The efficiency of the antigen ELISAs was evaluated by testing stool samples from three outbreaks (Table 2). Antigen ELISAs specifically detected antigenically homotypic strains.

Comparative capsid gene analyses of CHV and known NV strains indicated that CHV is most closely related to HV. The ORF2 genome of CHV and that of HV were identical in size, and showed 76% nucleotide identity and 85% aa identity. HV was first reported as the cause of an outbreak of gastroenteritis involving children and adults that occurred in Honolulu in 1971 (26). CHV was isolated in Japan 25 years after the isolation of prototype HV. Although CHV is genetically characterized as a HV-like virus, the cross-reactivity between CHV and the prototype HV by ELISA must be examined to determine the antigenic relationship between CHV and HV.

Based on phylogenetic analyses of RNA polymerase and capsid genes, NV strains have been classified into two major genogroups, genogroup I (GI) and genogroup II (GII) (17, 27). Recent detailed sequence comparisons demonstrated that each genogroup is further divid-

ed into genetic clusters (1, 7). When the capsid regions were compared, SEV formed one cluster with NV/68 and KY89, and CV was grouped into a new cluster within GI NV (Table 1 and Fig. 1). CHV belonged to GII NV. The capsid region of CHV showed 45% aa identity with both CV and SEV. CV and SEV have 71% aa identity in the capsid region. These three viruses were shown to be antigenically distinguishable from each other by type-specific antigen ELISAs. Based on these results, a good correlation between the antigenic typing by ELISA and genetic grouping in the capsid protein region was observed. Although further study is necessary to complete the antigenic discrimination among NV strains, expression of antigenically distinct VLPs and the subsequent preparation of hyperimmune sera to the VLPs will provide antigenic classification of NV strains. The unlimited supply of the antigen afforded by the use of a recombinant baculovirus and the preparation of antisera from antigenically distinct NV strains offers a stimulating approach to the genetic and antigenic study of these non-cultivable viruses.

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Empty Virus-like Particle-based Enzyme-linked Immunosorbent Assay for Antibodies to Hepatitis E Virus

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Hepatitis E, an enterically transmitted non-A, non-B hepatitis, is a serious viral infection that occasionally causes large epidemics in developing countries. In developed countries, the disease only appears sporadically due to the transmission routes, and it is considered to be less important. The hepatitis E virus (HEV) cannot grow in cultured cells and no reliable assay system has ever been developed. In addition, the present diagnostic are not perfect, and actual rates of HEV infection may be underestimated. Highly purified empty virus-like particles (VLPs) of HEV have been produced by the use of a recombinant baculovirus vector in insect cells. Using these VLPs as an antigen, an enzyme-linked immunosorbent assay (ELISA) for antibodies to HEV was developed. A panel of 164 sera that were randomized and coded, and sera collected periodically from three patients with hepatitis E were used for the evaluation. The sensitivity of the assay was shown to be equal to or better than that obtained in previous research that used the same serum panel. The ELISA demonstrated that the serum IgM level of the patients was highest at the onset of the clinical illness and then rapidly decreased. In contrast, a high level of circulating IgG antibody titers lasted for more than 4 years. In Japan, a non-endemic country, the prevalence of the IgG class antibody to HEV in healthy individuals was found to range from 1.9% to 14.1%, depending on the geographical area. Only one out of 900 (0.1%) serum samples was IgM-positive. The IgM class antibody to HEV was detected in 10.8% of non-A, non-B, and non-C acute hepatitis patients in northeast China, whereas none of the patients in Korea had the IgM antibody. The ELISA utilizing the VLPs is sensitive and specific in its detection of the IgM and IgG antibodies to HEV. The ELISA is therefore useful for diagnosing HEV infection and for seroepidemiological study of hepatitis E. ***J. Med. Virol. 62:327–333, 2000.***

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KEY WORDS: ELISA; recombinant HEV; VLP; anti-HEV IgM; anti-HEV IgG

INTRODUCTION

Hepatitis E virus (HEV) is the causative agent of the viral acute hepatitis formerly known as enterically transmitted non-A, non-B hepatitis [Bradley et al., 1987; Arankalle et al., 1988]. The virus spreads mainly by the fecal-oral route and is responsible for large epidemics and sporadic infections in developing countries [Wong et al., 1980; Khuroo, 1980; Balayan et al., 1983]. Because of its transmission routes, HEV can rarely cause large epidemics in developed countries, but it is responsible for imported acute hepatitis, that indicates that most people in developed countries do not have antibodies and are susceptible to HEV infection.

Hepatitis E was first recognized in 1980, and HEV was visualized by immune electron microscopy in 1983 [Balayan et al., 1983]. The development of effective diagnostic tests, however, has been hampered due to the lack of a cell culture system capable of growing HEV. Several recombinant antigen-based assays have been

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developed, and it is clear that, in HEV-endemic areas, 90–95% of the patients with acute hepatitis during outbreaks of hepatitis E develop anti-HEV antibodies [Favorov et al., 1992; Bryan et al., 1994]. Nonetheless, currently available assays still do not give a definite answer as to why a low level of anti-HEV has been observed consistently in non-endemic areas. A reliable antibody assay that is sensitive and specific to HEV infection has to be developed to study the seroprevalence of HEV infection in developed countries.

Recently, highly purified empty virus-like particles (VLPs) of HEV have been generated [Li et al., 1997)] and the VLPs seem to possess antigenicity similar to that of authentic HEV particles. The VLPs have been shown to function as a sensitive antigen for the detection of HEV-specific IgM and IgG by an ELISA [Li et al., 1997]. In this study the sensitivity and specificity of the ELISA for antibodies to HEV were evaluated using a panel of sera. The seroprevalence in healthy Japanese and in non-A, non-B, and non-C acute hepatitis patients in northern China and Korea was determined by the ELISA.

MATERIALS AND METHODS

Panel Sera

A panel of 164 randomized and coded sera tested previously to evaluate the performance of 12 different existing assays was used (Mast et al., 1998). The panel contained the following five sets of preparations: 1) early-convalescent sera collected from acute hepatitis E patients, and serial dilutions with normal human sera; 2) convalescent human sera obtained two months to 13 years after acute hepatitis E; 3) serial sera from chimpanzees taken one month to 3 years after inoculation with HEV from various geographic regions; 4) chimpanzee sera infected with hepatitis A virus, hepatitis B virus, or hepatitis C virus; and 5) human sera from United States blood donors.

Patient Sera

For the long-term tracing of anti-HEV antibodies, human sera were collected from three acute non-A, non-B, and non-C hepatitis patients. Patient A, a 57-year-old Japanese business man, had been hospitalized in Shanghai due to abnormal liver function and was then transferred to Japan for a closer medical examination. Patient B was a female Bangladeshi who developed acute hepatitis soon after entering Japan. Although the source of this infection was unknown, this was considered to be an imported case. Patient C was a 46-year-old male Japanese school teacher who had visited Beijing and Shanghai 4 months before the onset of acute hepatitis. He had brought back a liquid Chinese herbal medicine and took it for 6 weeks until the onset of the disease. HEV RNA was extracted from his plasma and amplified by RT-PCR. The nucleotide sequence analysis revealed a high degree of homology (99.8% of 752 nucleotides) with the Chinese strain, indicating that he may have been infected with HEV via the medicine (Ishikawa et al., 1995). These three patients were monitored for 20–55 months after the onset of hepatitis.

Patient sera from non-A, non-B, and non-C acute hepatitis were obtained from northeast China between 1986 and 1990, and also from Korea. The sera were tested for the anti-HAV IgM antibody, anti-HBs antigen, and anti-HCV IgG antibody using commercial diagnostic kits.

ELISA

To detect anti-HEV IgM and IgG using VLPs, an ELISA was established as follows: VLPs were expressed by a recombinant baculovirus as described previously [Li et al., 1997] (Fig. 1). Flat-bottom 96-well polystyrene microplates (Immulon 2, Dynex Technologies, Inc. Chantilly, VA) were coated with the purified VLPs (1μg/ml, 100 μl/well). The plates were incubated at 4°C overnight. Unbound VLPs were removed, and the wells were washed twice with 10 mM phosphate-buffered saline containing 0.05% Tween 20 (PBS-T), and then blocked for 1 hr with 200 μl of 5% skim milk (Difco Laboratories, Detroit, MI) in PBS-T at 37°C. After the plates were washed 6 times with PBS-T, human or monkey serum samples (100 μl/well) were added in duplicate at a dilution of 1:200 in PBS-T containing 1% skim milk. The plates were then incubated for 1 hr at 37°C. The plates were washed 6 times as described above and were administered with 100 μl of horseradish peroxidase-conjugated goat anti-human IgG (Cappel, West Chester, PA) (1: 10,000 dilution) or IgM (Cappel) (1:2,000 dilution) in PBS-T containing 1% skim milk. The plates were incubated for 1 hr at 37°C and washed 6 times with PBS-T. Then 100 μl of the substrate orthophenylenediamine (Sigma Chemical, Co., St. Louis, MO) was added to each well. The plates were incubated in a darkroom for 30 min at room temperature, then 50 μl of 4N H_2SO_4 was added to each well. After the plates had stood at room temperature for 10 min, the absorbance at 492 nm was measured.

RESULTS

Determination of Cut-Off Values in the Antibody ELISA

A panel of 164 sera, that had been randomized, coded, and used previously to evaluate 12 different assays for anti-HEV antibody [Mast et al., 1998], was employed to determine the cut-off values. Two human sera, six preinoculation chimpanzee sera, eight chimpanzee sera infected with hepatitis A virus, hepatitis B virus, or hepatitis C virus, and 35 sera from blood donors were selected. These 51 sera were previously shown to be negative for anti-HEV IgG in two EIA employing the capsid proteins expressed in insect cells through the use of recombinant baculovirus (Tests 1 and 2) [Mast et al., 1998]. The sera were also negative for anti-HEV IgG in 9 out of 10 tests employing recombinant capsid proteins from *E. coli* or synthetic peptides as described previously [Mast et al., 1998]. OD values ranging from 0.009–0.117 were obtained from 51 sera; an OD value of 0.150 calculated using four

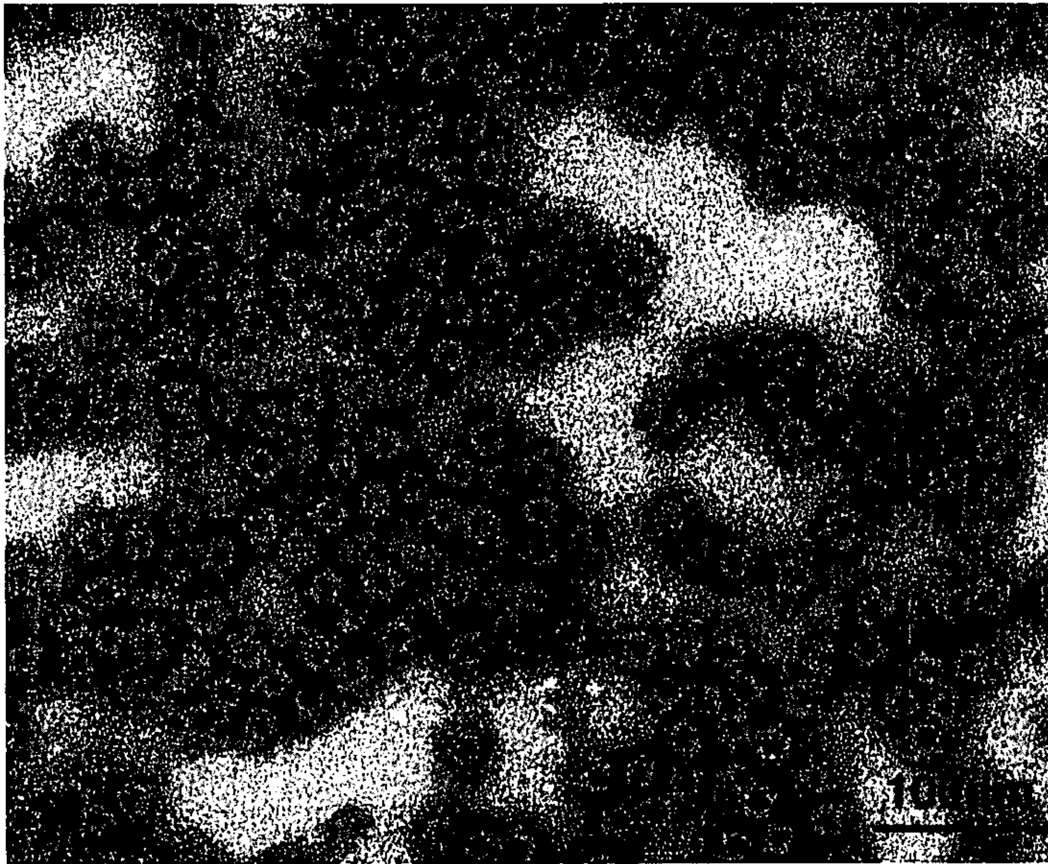

Fig. 1. Electron micrograph of the purified HEV VLPs. The diameter of the VLPs is 23.7 nm.

standard deviations (0.026 × 4 = 0.104) above the mean value (0.046) was employed as the cutoff value of the IgG. To determine the cut-off value of the IgM, 86 negative sera for anti-HEV IgG were collected from patients with chronic hepatitis C in Japan and used as a panel. The OD values ranged from 0.008 to 0.097, and four standard deviations above the mean value, 0.110, was used as the tentative cut-off value. Using this cut-off value, the panel of 164 sera were tested. Of these, 154, including 51 negative sera for anti-HEV IgG, were shown to be negative for anti-HEV IgM. Therefore, the cut-off value for the IgM was calculated again using the 154 sera. The same OD value, 0.110, was obtained and used as the cut-off value of the IgM. The assays were done at least in triplicate to confirm reproducibility.

Sensitivity and Specificity for the Detection of IgG Antibody

The sensitivity of the ELISA for detecting the IgG was evaluated from the end-point serum dilution (Table I). The absorbance of the IgG-positive serum decreased proportionally with dilution, and the end-point was shown to be 1:160. This dilution factor is the same as that reached by Test 2 in the previous study and higher than that reached by Test 1 in that study [Mast et al., 1998]. In that study, both tests 1 and 2 were EIA using recombinant proteins expressed in insect cells.

TABLE I. Sensitivity of the ELISA Using HEV VLPs

Serum dilution	OD[a]	Test 1[b]	Test 2[c]
1:5	2.857 (+)	+	+
1:10	2.087 (+)	+	+
1:20	1.260 (+)	+	+
1:40	0.625 (+)	+	+
1:80	0.379 (+)	+	+
1:160	0.196 (+)	−	+
1:320	0.115 (−)	−	−
1:640	0.080 (−)	−	−
1:1280	0.043 (−)	−	−

[a]Positive (+) or negative (−) by ELISA.
[b]Result from Test 1 described in Mast et al., 1998.
[c]Result from Test 2 described in Mast et al., 1998.

To determine further the sensitivity for the detection of the IgG antibody, five sera from patients with acute hepatitis and 24 sera from chimpanzees inoculated with HEV strains from various geographic regions were tested. These 29 samples were shown previously to be positive for anti-HEV IgG by both Tests 1 and 2 [Mast et al., 1998]. The anti-HEV antibody titers of the patients ranged from >3.2 OD for a pooled serum collected 2–4 months after onset to >2.7 for a serum collected more than 13 years after onset. The OD values of 22 of the 24 chimpanzee sera ranged from >3.4 at 32–

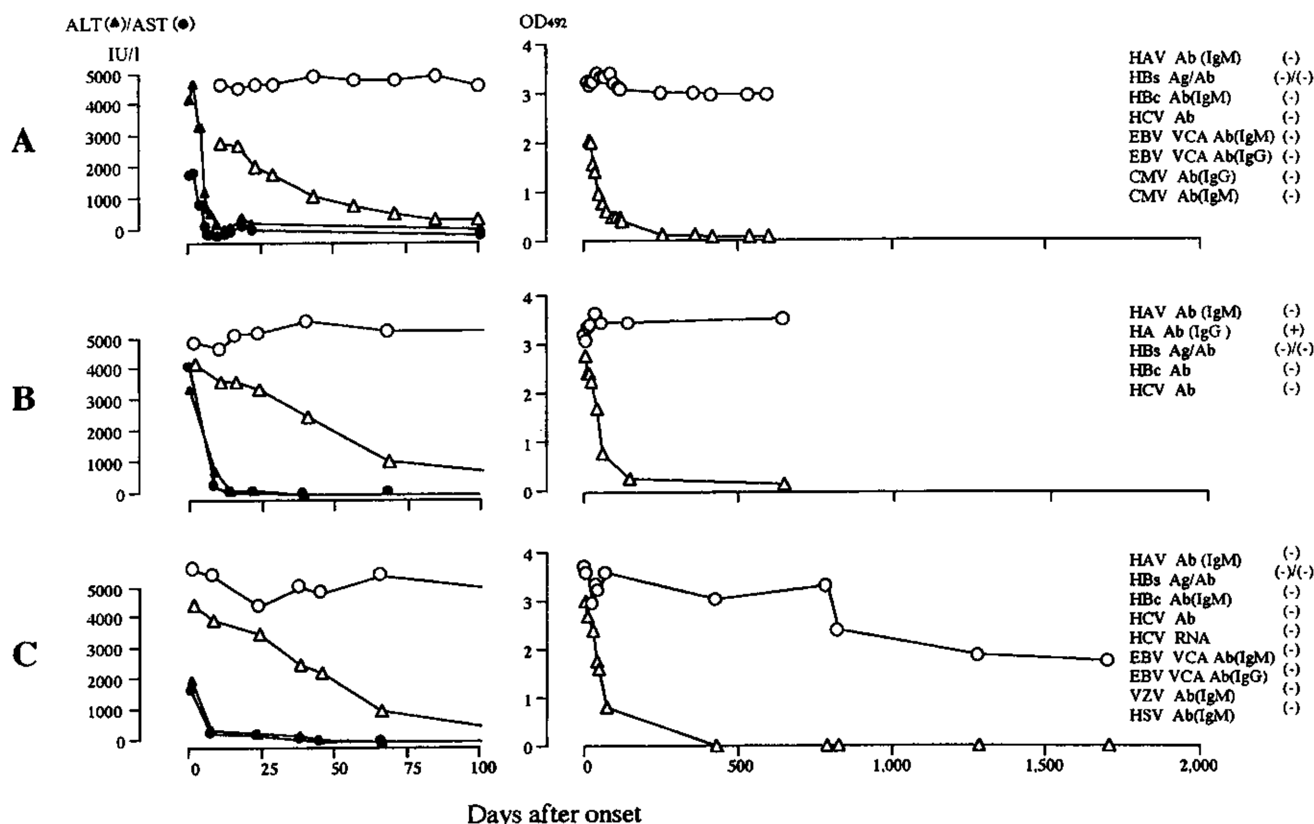

Fig. 2. Detection of anti-HEV antibodies in sera from hepatitis E patients. Sera were periodically collected after onset, and the IgM (△) and IgG (○) antibodies were measured using the ELISA as described in Materials and Methods. The short-term period (**left panels**) with serum enzymes, ALT (alanine aminotransferase) (▲), and AST (asparate aminotransferase) (●) and long-term period (**right panels**) are shown separately. Abbreviations: HAV, hepatitis A virus; HBsAg, hepatitis B virus surface antigen; HBcAb, hepatitis B virus core antibody; HCV, hepatitis C virus; EBV VCA, Epstein-Barr virus core antigen; CMV, cytomegalo virus; HSV, herpes simplex virus; VZV, varicella zoster virus.

39 days after infection to >0.6 at 3 years after infection. Two chimpanzee sera showed results discordant with the previous study. These were negative for anti-HEV IgG by Test 1 but positive for IgG by Test 2, and had OD values of 0.254 at 2 years after infection and 0.245 at 3 years after infection. Thus all 29 samples were positive for anti-HEV IgG and the results were exactly the same as those obtained by Test 2. In contrast, six preinoculation chimpanzee sera, eight sera from chimpanzees infected with hepatitis A virus, hepatitis B virus, or hepatitis C virus, and two normal human sera (24 samples total) were all found to be negative for IgG by the assay.

A further test was done using a panel of human sera from U.S. blood donors. In the previous study, 39 out of the 101 sera were shown to be negative for anti-HEV IgG by at least 2 assays (Tests 6 and 7) [Mast et al., 1998]. Both assays were EIA using recombinant proteins expressed in *E. coli*. It seemed that 35 out of 39 specimens showed the same negative results for anti-HEV IgG. Four specimens, however, were shown to be positive for the IgG. To clarify the discrepant results, the reactivity of HEV VLPs was tested by Western blot analysis, and three of the four specimens showed a positive reaction (data not shown). As the OD value of one of them exceeded 0.5, and the four specimens were shown to be positive for anti-HEV IgG by Test 2 in the previous study, they are likely to be antibody-positive. In addition, out of 17 sera that were shown to be positive for anti-HEV IgG by at least 3 assays (Tests 1, 6 and 7) [Mast et al., 1998], one specimen was found to be negative. This sample was also negative by Western blot analysis (data not shown). Also, 45 sera with concordant results in prior assays were clearly separated into 33 anti-HEV-positive and 12 anti-HEV-negative sera. As a result, the 48 sera from the blood donors that had been found to be positive for IgG by Tests 1 and 2 were all found to be positive for anti-HEV IgG. The results obtained in this study using the panel of 164 sera were exactly the same as those from Test 2 in the evaluation study [Mast et al., 1998].

Antibody Responses in HEV Patients

Figure 2 shows the anti-HEV IgG and IgM antibody profiles associated with the HEV infection. Test sera were collected periodically from three patients with hepatitis E as described in Materials and Methods. The IgM antibody levels were the highest at the onset of the disease, and then they decreased rapidly in all patients. The IgG antibody level was as high as 3.0 OD at

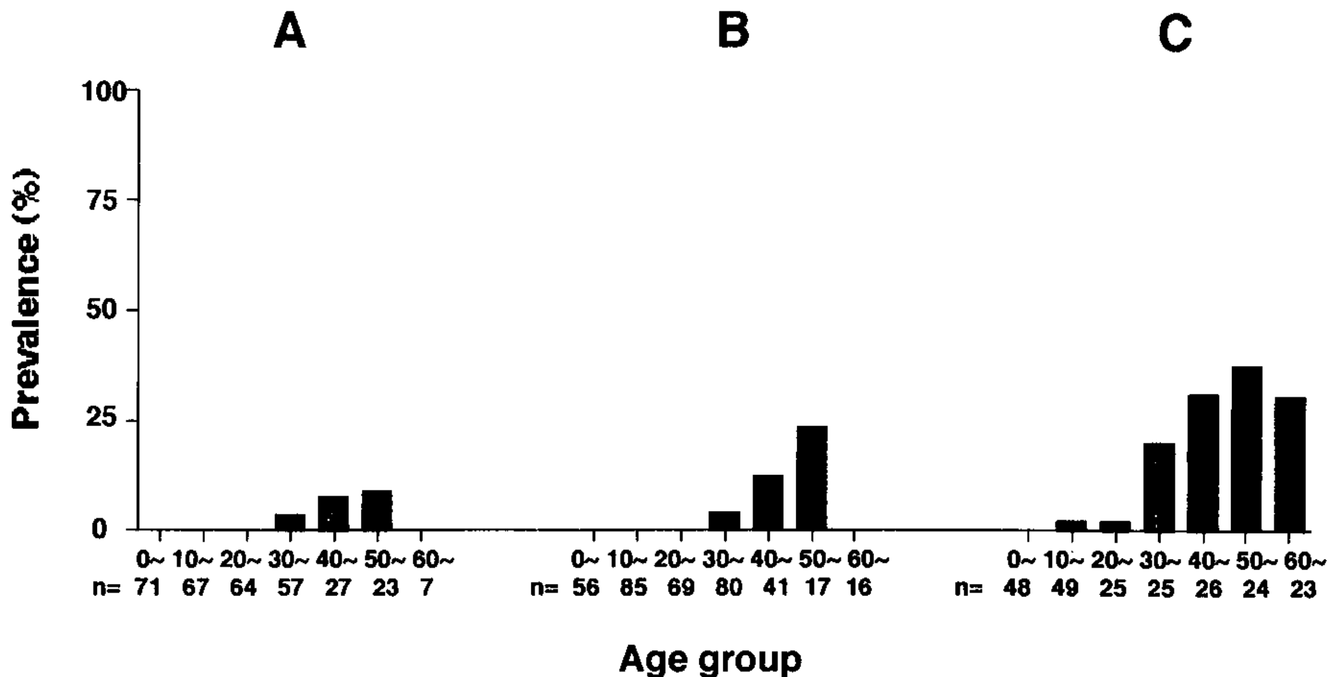

Fig. 3. Age-specific prevalence of anti-HEV IgG antibody was determined using a total of 900 sera from healthy Japanese, including 316 sera from a southern prefecture (**A**), 364 from a northern prefecture (**B**), and 220 from a central prefecture (**C**).

the onset in all three patients and persisted at high levels. In Patient A, the IgM antibody was detectable until 112 days after the onset of clinical illness but seems to have disappeared shortly after this point. The antibody was undetectable at 8 months after onset. The OD values of the IgG were still close to 3.0 at 600 days after onset in Patient A. Similar patterns were observed in Patients B and C, with the IgM antibody testing positive with OD values of 0.26 at 154 days in Patient B and 0.80 at 66 days in Patient C. There was no discernible decrease in the IgG antibody until Day 643 in Patient B and Day 785 in Patient C. Relatively high OD values of the IgG antibodies (>1.5) lasted for a long time, and this OD level was still detectable after 4 years and 7 months (1,699 days) in Patient C. A similar long-term IgG antibody level has been observed in the sera of an experimentally infected monkey as shown previously [Li et al., 1997].

Antibody Positive Rate in Healthy Japanese

The age-specific seroprevalence of anti-HEV IgG and IgM was measured using 900 sera collected from healthy individuals who live in relatively small areas in the northern, central, and southern prefectures of Japan (Fig. 3). None of the IgG-positive sera was found in patients under the age of 29 in the northern and southern prefectures (Fig. 3A,B), and only two out of 534 (0.4%) sera samples in the central prefecture were IgG-positive in this age group (Fig. 3C). The prevalence of the antibody to HEV in patients over age 30 was age-related and increased in a cumulative fashion in all three geographical areas. The prevalence of the IgG antibody in the southern prefecture increased from 3.5% in the 30–39-year-old group to 8.7% the 50–59-year-old group (Fig. 3A). The average positive rate was 1.9% (six out of 316 samples) in the southern prefecture. A similar prevalence rate was observed in the northern prefecture, ranging from 3.8% in the 30–39 age group to 23.5% in the 50–59 age group with an average 3.3% (12 out of 364 samples) (Fig. 3B). The IgG-positive rates of the central prefecture, however, were different. There, the rate increased moderately with age. A rate as high as 37.5% was found in the 50–59 age group (Fig. 3C). The average rate, 14.1% (31 out of 220 samples), was approximately 4 to 7 times higher than that of the other two prefectures, and these differences are statistically significant ($P < 0.001$, χ^2 tests). The OD values of 5 out of 31 IgG-positive sera in the central prefecture were close to the cutoff value, whereas the OD values of the other 26 samples evenly ranged from 0.215 to 1.106. In contrast to the relatively high IgG-positive rate, only one individual out of 900 (0.1% of the total) was found to be IgM-positive in the southern prefecture. As the OD values of the IgG and IgM were 0.899 and 0.359, the infection seems to be asymptomatic.

Hepatitis E in Non-A, Non-B, and Non-C Hepatitis Patients

Using 65 sera from non-A, non-B, and non-C acute hepatitis patients, the prevalence of the IgM-class antibody to HEV was measured to evaluate HEV infection in China. Seven out of 65 (10.8%) individuals were positive for anti-HEV IgM (Table II). Twenty-three out of 65 (35.4%) patients, including the seven patients with the anti-HEV IgM antibody, appeared to be posi-

TABLE II. Anti-HEV IgG and IgM in Non-A, Non-B and Non-C Acute Hepatitis Patients in China and Korea

		Anti-HEV IgG	Anti-HEV IgM
Endemic area	(China)	23/65 (35.4)*	7/65 (10.8)
Non-endemic area	(Korea)	17/42 (40.5)	0/42 (0)

*Number positive/number tested (%)

tive for IgG. In contrast, none of the 42 sera from non-A, non-B, and non-C acute hepatitis patients in Korea were positive for anti-HEV IgM, although the IgG-positive rate (40.5%) of these patients was similar to that of the patients from China ($P > 0.10$, χ^2 tests).

DISCUSSION

Recently, 12 available assays for antibodies to HEV were evaluated collaboratively using a common serum panel. The high-titer sera obtained in the endemic area showed consistent and reliable results by most of these assay systems. When 101 serum samples from blood donors in the USA were tested, however, discrepancies in the different systems appeared, and it was concluded that none of these assays can be applied universally for the detection of specific antibodies to HEV due to their non-specific reactions and to differences in their sensitivities [Mast et al., 1998]. One reason for these inconsistencies may rest with the antigenic varieties in the HEV strains, although this is unlikely because the capsid protein of HEV was broadly reactive [Tsarev et al., 1993; Meng et al., 1997, 1998a,b; Thomas et al., 1997].

The best antigen for the detection of antibodies to HEV is native HEV. HEV cannot grow in vitro, however, and not many native infectious HEV particles are available. Several attempts to express the capsid protein of HEV have been made, but none of the systems tried have succeeded in yielding a uniform, large amount of VLPs [He et al., 1993; Tsarev et al., 1993; Li et al., 1994; Panda et al., 1995; McAtee et al., 1996; Zhang et al., 1997]. HEV VLPs have been generated by expressing the truncated ORF2 at its N-terminus of the genome through the use of recombinant baculovirus in insect cells [Li et al., 1997]. The advantage of this system is not only its high yield (1.0 mg/10^7 cells) but also its efficient release into the culture fluid, making it possible to prepare a milligram order of VLPs. Furthermore, the VLPs used in this study were shown to have similar antigenicity, as shown by the serum IgM and IgG of experimentally infected monkeys both being detectable [Li et al., 1997]. The overall sensitivity and specificity of the ELISA seemed to be the same as those shown previously (Test 2) [Mast et al., 1998], probably because both the present and previous assays used the baculovirus-expressed capsid proteins. The serological tests with sera collected from hepatitis E patients indicated that the circulating IgG antibody was maintained at a relatively high level.

The relatively low positive rate of circulating anti-HEV antibody in the populations of the endemic areas combined with the unexpectedly higher prevalence of anti-HEV in non-endemic countries make interpretation difficult [Dawson et al., 1992; Lok et al., 1992; Paul et al., 1994; Zanetti and Dawson, 1994]. Because of the lack of reliable antibody detection assays, the significance of HEV infection, that is without doubt a serious infection in developing countries, might be underestimated in developed countries. Recently, novel strains of HEV were identified in industrialized countries including United States, Italy, Greece and Taiwan in patients who have no history of travel to hepatitis E endemic countries [Schlauder et al., 1998; Erker et al., 1999; Schlauder et al., 1999; Hsieh et al., 1999]. In addition, swine HEV that is very close genetically to human HEV has been reported (Meng et al., 1997, 1998a,b; Schlauder et al., 1998]. Recently, swine HEV appeared to have the ability to cross species and to be capable of infecting rhesus macaques and chimpanzees [Meng et al., 1998b], a finding that suggests that this HEV strain may be capable of infecting humans. HEV antibodies have been detected in pigs, lambs, and cows, and more than 80% of the rats in the United States have been shown to have HEV antibodies. All of these results suggest that many animals worldwide are infected naturally by HEV and that zoonotic infection may occur.

Although the prevalence of the IgG in healthy Japanese varied from 1.9–14.1% depending on the region, the overall IgG antibody positive rate is equivalent to or higher than the rate in India, where HEV is endemic [Arankalle et al., 1995]. It is generally thought that HEV is not endemic in Japan. Indeed, acute hepatitis due to HEV is very rare, and most hepatitis E patients are imported cases. Detection of HEV antigens or virus genomes in human specimens is needed to clarify the low level of the antibody positive rates.

The anti-HEV IgG positive rates of non-A, non-B, and non-C acute hepatitis patients seemed to be similar in both non-endemic and endemic areas (40.5% in Korea and 35.4% in China), although the IgG positive rates of these hepatitis patients were higher than those of the normal population. The prevalence of anti-HEV IgM antibody indicated that hepatitis E is not endemic in Korea but is prevalent in China.

It should be noted that ELISA titers in healthy Japanese, that showed ODs of 0.359 in anti-HEV IgM and ODs of 0.185 to 1.106 in anti-HEV IgG, were low compared with those observed in acute hepatitis E patients, where ODs >2.0 for IgM and >3.0 for IgG have been indicated as described in Figure 2 and in the previous paper [Li et al., 1997]. These results suggest that the onset of acute hepatitis is somehow linked to the high antibody titers.

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