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LEGENDS TO FIGURES

FIGURE 1: Panel A: Effect of DNA dosage on anti-ORF2 antibodies. Groups of five mice were given intramuscular injection of 1, 10, 50 or 100 μg of pcDNA-ORF2. Sera were collected and analyzed for the presence of anti-ORF2 antibodies by EIA. The figure shows the level of total IgG antibodies after 8 weeks of injection and is an average of duplicate absorbance (Optical Density, O. D.) values on pooled sera obtained after subtracting the values obtained with pre-immune sera (O.D. of 0.05).

Panel B: Longevity of immune response: Groups of five mice were injected with 100 μg of pcDNA-ORF2 and boosted 4 weeks later with same amount of DNA. Sera were collected at different time intervals and analyzed for the presence of anti-ORF2 antibodies with ELISA. The data are presented as mean ± SD for five animals per time point.

FIGURE 2: IL-2 expression plasmid co-injected at different times modulates the antibody responses. Panel A: Groups of mice were injected with pcDNA-ORF2 (closed circles, group A), pcDNA-ORF2 and pIL-2 at day zero (open circles, group B) and pcDNA-ORF2 and pIL-2 injected after 4 days of injection of pcDNA-ORF2 (triangles, group C). The arrow indicates the time of boost. The anti-ORF2 antibodies were measured in the pooled sera by EIA. Values shown are a mean of two separate experiments.

Panel B: IgG isotype profile in the sera of mice immunized with pcDNA-ORF2 and pIL-2 injected after 4 days of injection of pcDNA-ORF2 (group C of figure 2). Anti-ORF2 specific total IgG, IgG1, IgG2a and IgG2b antibodies were detected by EIA with isotype-specific secondary antibodies. The data are presented as mean ± SD for five animals.

FIGURE 3: Effect of co-injection of GMCSF expression plasmid on antibody responses. Panel A: Groups of five mice were injected with pcDNA-ORF2 alone (closed circles) and pcDNA-ORF2 and pGM-CSF (open circles). The arrow indicates the time of boost. The anti-ORF2 antibodies were measured in the pooled sera by EIA. Values shown are a mean of two separate experiments.

Panel B: IgG isotype profile in the sera of animals immunized with pcDNA-ORF2 and pGM-CSF. Anti-ORF2 specific total IgG, IgG1, IgG2a and IgG2b antibodies were detected by EIA with isotype-specific secondary antibodies. The data are presented as mean ± SD for five animals.

FIGURE 4: Panel A: Kinetics of appearance of anti-ORF2 antibodies. The figure shows the antibodies in groups of animals immunized with pcDNA-ORF2 alone (closed circles) or in animals primed with pcDNA-ORF2 and boosted with 10 μg of ORF2 VLPs (open circles). Arrow A indicates the time of pcDNA-ORF2 boost to both the groups and arrow B indicates the time of protein boost to one group only. The anti-ORF2 specific IgG antibodies were detected by EIA. Four mice in each group were used and the results are a mean of two different experiments.

Panel B: IgG isotype profile in the sera of animals primed with pcDNA-ORF2 and boosted with VLPs. Anti-ORF2 specific total IgG, IgG1, IgG2a and IgG2b antibodies were detected by EIA with isotype-specific secondary antibodies. The data are presented as mean±SD for four animals.

4

Empty Virus-Like Particles of Hepatitis E Virus Expressed by a Recombinant Baculovirus

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INTRODUCTION

Hepatitis E virus (HEV) is one of the main causative agents of acute viral hepatitis formerly known as an enterically transmitted non-A, non-B hepatitis. Like hepatitis A virus, HEV is mainly transmitted by fecal-oral routes and large epidemics due to this virus are often associated with contaminated water (1, 2). Table 1 illustrates several historical epidemics reported so far. They are mainly Asian countries, but hepatitis E is a serious public health problem in most developing countries throughout the world. Previous reports indicated that anti-HEV was detected in 1–2% of the population in developed countries, while in those countries such as India (4–20%), Mexico (7.5–19.5%) and Egypt (12.5–23.5%) prevalence rates were high. However, those epidemiological data were all derived from antibody assays using different HEV antigens with different sensitivities. To determine the global level-prevalence of HEV infection, simple comparisons of previous results is almost meaningless. It is suggested that HEV infection mainly proceeds without showing a manifestation of clinical symptoms (3). Although there are at least 3 known genotypes, HEV may have only one serotype with the wame antigenicity (Fig. 1). It is thus very important to perform a well arranged seroepidemiology of HEV infection by using adequate HEV antigens.

Table 1. Big epidemics of hepatitis E

	Year	No. of patients
Kyrgyzstan	1955-56	10,800
New Delhi	1955-56	2-40,000
Kathmandu	1973-87	10,000 × 3
Myanmar	1976-79	40,000
Xinjiang	1986-88	120,000
Borneo	1987, 1991	4-5,000 × 2
Kanpur	1991	79,000

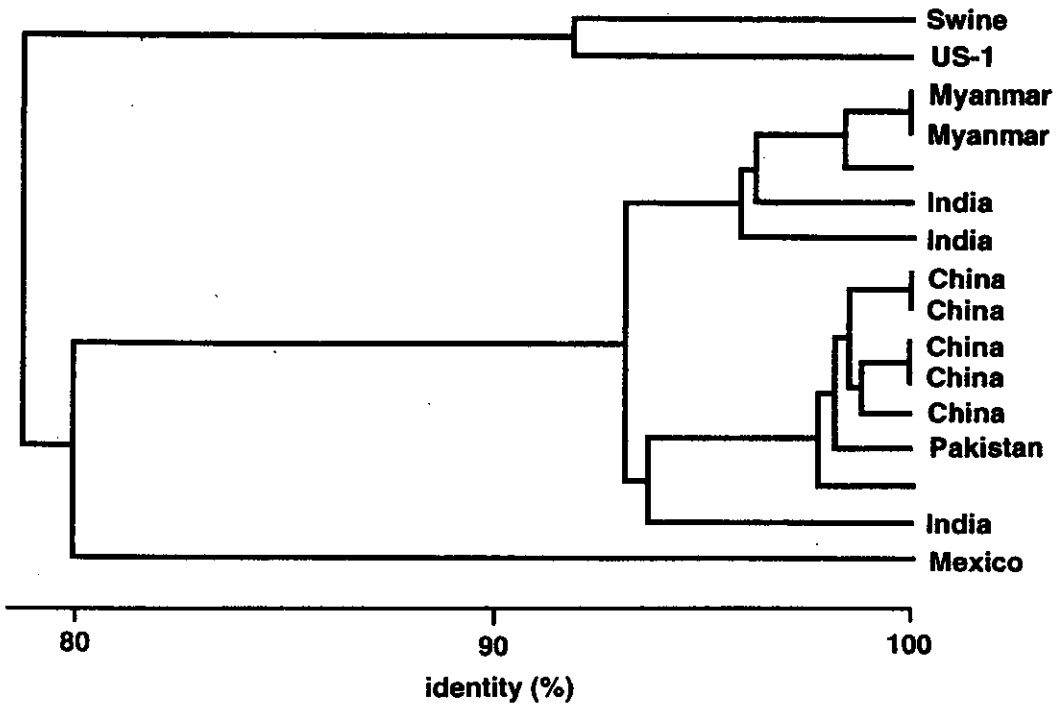

Fig. 1. Phylogenetic tree based on the nucleotide sequences of ORF2.

CHARACTERIZATION OF HEV

HEV is a non-enveloped, small RNA virus with a diameter of about 30 nm. Virus particles with 27-34 nm are detected from feces of patients or experimentally infected monkeys. The morphology of HEV particles in an electron microscope is similar to that of small round-structural virus (SRSV) which is the main cause of non-bacterial diarrhoeal disease. And it was previously tentatively classified as *caliciviridae*. However, as is shown later, the organization of the HEV genome, in particular its domain encoding nonstructural proteins, rather resembles togaviruses like rubella virus. Consequently the classification of HEV is not completely settled yet.

There is no tissue culture cell system to support the replication of HEV yet. Human being is the only susceptible animal to develop hepatitis. But experimentally, chimpanzees, tamarines, green monkeys, rhesus monkeys, and cynomolgus monkeys are also susceptible. Recently, a new virus in swine was shown to be related to HEV (4). This suggests an important possibility that hepatitis E is a zoonosis, but details have to be elucidated.

HEV GENOME

HEV contains a single-stranded, approximately 7.5 kb RNA molecule that is 3′ polyadenylated and includes three open reading frames (ORFs) (Fig. 2). The N-terminus ORF1 is considered to encode viral nonstructural proteins. Their individual functions are not proved as yet, but from their relative position and some consensus key sequences, they are considered to be methyltransferase, papain-like cystein proteinase, RNA helicase, and RNA dependent RNA polymerase. The second ORF (ORF2) located at the C-terminus of the genome encodes a 71 kDa protein for the putative viral capsid. It has an 18- hydrophobic amino acid cluster at its N- terminus which is considered as signal peptides. The third ORF (ORF3), with unknown function, is mapped between ORF1 and ORF2 (5–11).

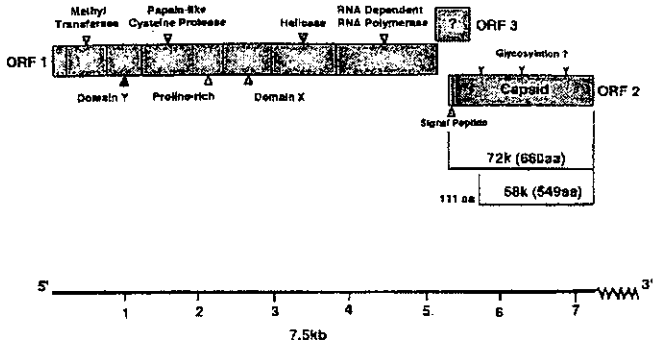

Fig. 2. Functional domains of HEV ORFs.

EXPRESSION OF THE HEV CAPSID PROTEINS

Ever since HEV was first recognized in 1980 and visualized by immune electron microscopy in 1983, many efforts have been made to express the structural protein as empty virus-like particles (VLPs) (12–14). This is particularly important because no practical cell culture system has been available to allow the growth of HEV. In fact recombinant structural proteins have been expressed efficiently by *E.coli* (15–17) or insect cells (14, 18), and less efficiently by yeast or mammalian cells (13). However, as either full length- or truncated forms, no efficient system was achieved to generate a considerable amount of viral particles suitable for virological characterization and for practical use for diagnostics.

Expression of the structural proteins of a Myanmer strain of HEV was carried out using a recombinant baculovirus harboring the entire ORF2, in two different insect cell lines, Sf9 and Tn5. cDNAs used for expression experiments are summarized in Fig. 2. Three major proteins with molecular weight (MW) of 72, 58 and 50 kDa were observed in cell lysates from these two cell lines (Fig. 3). A protein band with an MW of 72 kDa is thought to be a primary translation product encoded by the ORF2 and is in agreement with the MW calculated from its aa sequence (71 kDa). All these three proteins were immunoreactive with antibodies in serum sample from a patient with acute phase hepatitis E as assayed by Western blot analysis, indicating that they share the same antigenic epitopes. The 72kDa protein, as previously indicated by others (12,18,19), appeared to be tightly cell-associated and was difficult to be dissolved. The 58 and 50 kDa proteins accumulated in infected cells at a later stage of the infection. Almost identical results were obtained in both Sf9 and Tn5 cells. However, overall expression of these proteins, particularly 72 kDa protein, seemed higher in Tn5 cells, and none of these proteins self-assembled into VLPs. These findings are basically consistent with the results described previously by others (13, 20).

In an attempt to express the capsid protein as VLPs, we then prepared several mutant baculoviruses to express modified ORF2 proteins at both N- and C-teminus. Both Sf9 and Tn5 cells were infected with the recombinant baculovirus that contains a N-terminus 111-aa-truncated ORF2, Ac5480/7126. In both cells two to three major immunoreactive proteins including MW of 58 and 50 kDa were found. These proteins were only detected in the recombinant virus-infected cells, but not in mock-infected or wild-type baculovirus-infected cells. The 58 kDa protein was in agreement with the MW (59 kDa) calculated from the aa 112-660 of the truncated ORF2 of the Myanmer strain. In both cells, the 58 kDa proteins were tightly cell-associated and never observed in the culture medium. In contrast to the culture medium from the Sf9 cells where no protein was detected, an intense band with an MW of 50 kDa was observed

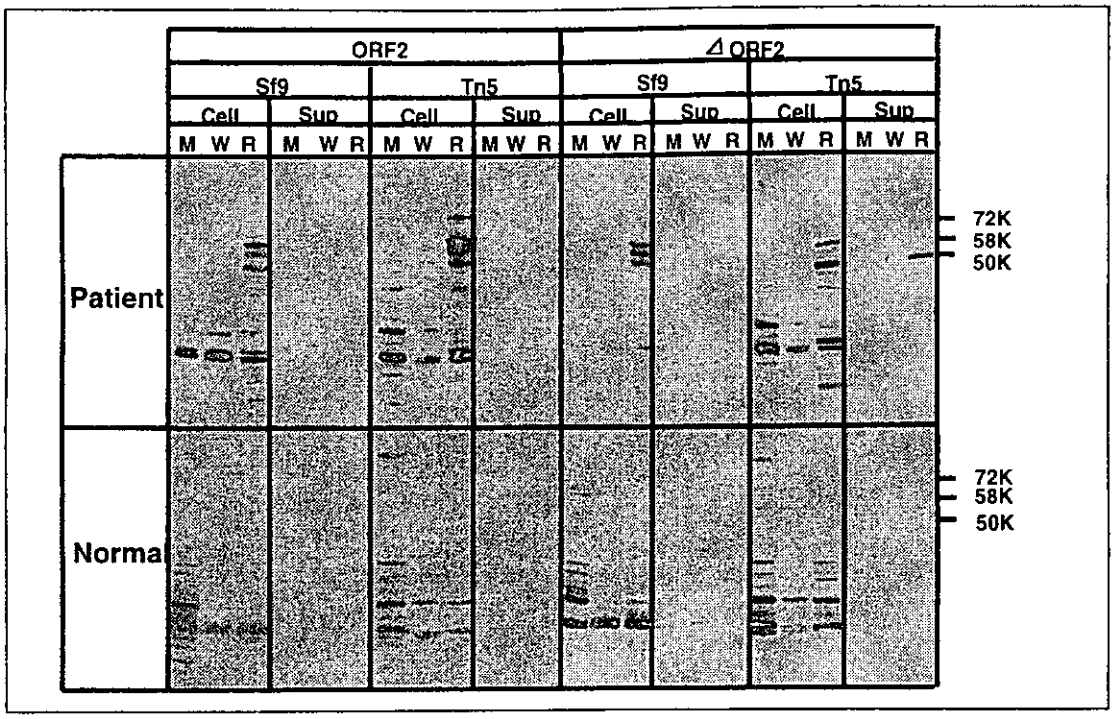

Fig. 3. Western blot assay of entire (ORF2) and truncated (ΔORF2) capsid proteins in insect cells.

in the culture medium from Tn5 cells. Interestingly, in Tn5 cells, approximately 90% of the 50 kDa protein was recovered from the medium.

The supernatant of the recombinant virus-infected Tn5 cells was clarified by centrifugation and then concentrated by PEG 6,000. The 50 kDa protein was exclusively recovered from the large precipitate, suggesting that the proteins were aggregated or self-assembled to form empty particles. Surely many round-shaped, empty virion-like particles were observed by EM. Most were in aggregated form. The size was estimated to be 23.7 nm in diameter using 100 particles for the calculation. This size is a little smaller than 27 nm of native HEV particles secreted into the bile or stool of experimentally infected monkeys.

A major peak of 50 kDa protein was detected at a density of 1.285 g/cm^3. Although the buoyant density of HEV particles in stool extract obtained from a human volunteer was reported to be 1.35 g/cm^3 and 1.39-1.40 g/cm^3 in CsCl, the density of native empty virus is yet unknown. The amino acids sequence of the N-terminus analyzed by Edman automated degradation revealed that the first 15 aa of the 50 kDa protein were identical to the 15 aa between 112 and 126 of the ORF2. This aa sequence is also identical to the N-terminus of the 58 kDa primary translation product predicted from the plasmid construction. This indicates that the 50 K protein was generated from the 58 kDa protein by a post-translational modification, probably by the cleavage(s) at its C-terminus.

The intracellular 50 kDa protein of infected Tn5 cells also contains the same N-terminus amino acid sequence as 50 kDa proteins in the medium and the protein was recovered as soluble form after three cycles of freezing-thawing. Therefore the physicochemical properties of the intra- and extra cellular 50 kDa proteins in the Tn5 cells are considered to be the same.

ANTIGENICITY OF THE VLPs

The 50 kDa VLPs were then mixed with the diluted serum from a patient with hepatitis E, and examined under EM. The VLPs were coated with specific antibodies forming massive aggregations. To further examine the antigenic specificity, the VLPs were used as antigens to coat microplates to detect HEV specific-antibodies elicited in the serum of the experimentally infected monkey as depicted in Fig. 4. Significant increase of both IgM and IgG antibody responses was observed during the clinical course of the acute hepatitis. The IgM antibody appeared soon after the onset, reached to the highest after 4 weeks and immediately decreased. There was no detectable IgM after 12 weeks. The IgG response rapidly increased and reached to the peak at 13 weeks after inoculation. The level of IgG antibody decreased gradually but the antibody level over 0.5 OD as measured by ELISA lasted for at least 2 years. These results indicated that VLPs obtained from the supernatant of infected Tn5 cells were antigenically similar to the native HEV. It is expected to serve as an antigen to detect specific antibodies against native HEV.

IMMUNOGENICITY OF THE VLPs

Highly purified 50 kDa VLPs were used to immunize rabbits and guinea pigs. After four injections these animals elicited high level of antibodies and the titer reached as high as $1:10^6$ as assayed by the antibody ELISA. To evaluate immunogenicity of VLPs the hyperimmune sera from rabbit were coated on the microplate and HEV antigens then captured by the antibodies were detected. Diluted biles from pre- and post HEV-infected monkeys were used as antigen and a hyper immune serum from guinea pig as a detector antibody. The titers 1:400–1:800 were obtained. These results suggested that VLPs could elicit antibodies which could bind to native HEV antigens.

DISCUSSION

Recently Jameel and his co-workers reported the expression of the entire ORF2 protein in COS-1 cells using simian virus 40-based expression vector. They showed that ORF2 protein is synthesized as a 82 kDa precursor which is then processed into the 74 kDa mature protein through signal sequence cleavage and then glycosylated into 88 kDa protein. This glycoprotein has the potential to form noncovalent homodimers (21). However, the particle formation is not known. We also do not know whether or not the capsid protein of the native HEV virion is glycosylated. Therefore, there is a possibility that VLPs described here might be an artefact produced by an unknown assembly mechanism specific to Tn5. Our preliminary immunoblot analysis identified two major proteins with MW of 50 and 58 kDa in the biles and stools from experimentally infected monkeys (data not shown). However, again we do not know whether or not one or both of these two molecules constitute the authentic HEV virion because neither the MW nor the N- and C-termini aa sequences of the capsid protein of the native HEV virion has been reported. Sequence analysis of both N- and C-terminus of native HEV capsid protein is required to ascertain this issue.

The empty particle formation of HEV, nevertheless, described here is very important. The VLPs appeared to retain the antigenicity of the native virion. Although the neutralization test with the authentic HEV particles is necessary to evaluate the immunogenicity (22), our results suggests that VLPs could be the most promising molecules for a recombinant vaccine against HEV (22).

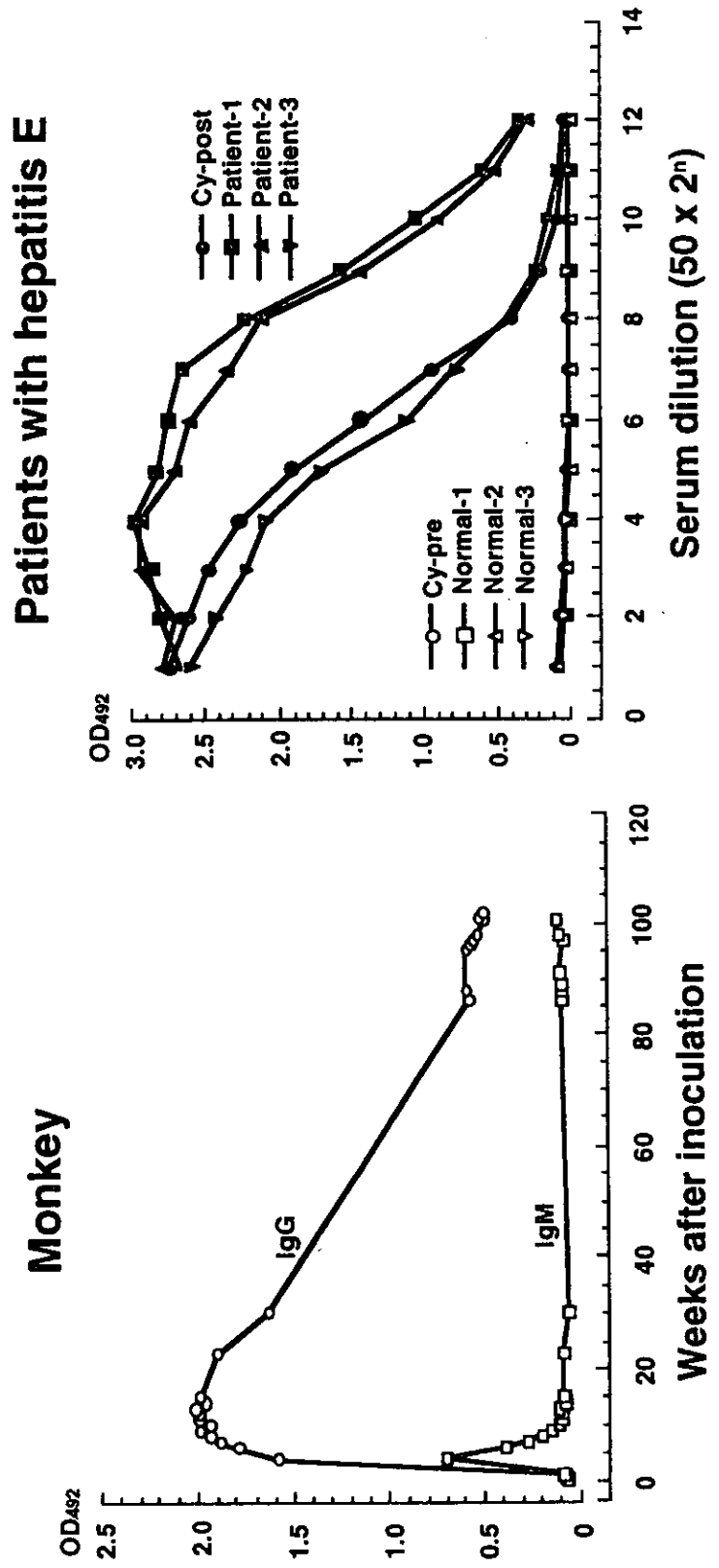

Fig. 4. Anti-HEV antibody responses.

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Molecular Cloning, Expression and Antigenicity of Seto Virus Belonging to Genogroup I Norwalk-like Viruses

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Running title: Antigenicity of recombinant Capsid protein of Seto virus

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ABSTRACT

The viral capsid protein of the Seto virus (SeV), a Japanese strain of genogroup I Norwalk-like viruses (NLVs), was expressed as virus-like particles using baculovirus expression system. An antigen-detection ELISA based on hyperimmune antisera to recombinant SeV was highly specific to homologous SeV-like strains, but not to heterologous strains in stools, allowing us type-specific detection of NLVs.

Norwalk-like viruses (NLVs), one of the four genera in the family *Caliciviridae*(3), are a genetically and antigenically heterogeneous group of viruses that are a major cause of outbreaks of acute nonbacterial gastroenteritis (1,13). Detection and molecular characterization of NLVs have been hampered by a lack of cell culture systems and small animal models. However, recent progress in the molecular cloning and sequencing of RNA-dependent RNA polymerase and capsid protein genes has enabled us to divide NLVs into at least two genogroups: genogroup I (GI) and genogroup II (GII) (22).

NLVs contain a single-stranded positive-sense RNA genome that encodes three open reading frames (ORFs) (12, 17). When the ORF2 gene is expressed by a recombinant baculovirus, the recombinant protein spontaneously self-assembles into virus-like particles (VLPs) that are antigenically and morphologically indistinguishable from native virion (4, 5, 6, 9, 11). The VLPs have been successfully used in structural studies (19, 20) as well as in the development of enzyme-linked immunosorbent assays (ELISAs) for serological diagnosis of NLVs infection (4, 5). Though antigen-detection ELISAs using hyperimmune antisera raised against the VLPs have been developed to detect NLVs in stools (2, 6, 8), the efficiency is relatively low due to the antigenic diversity of NLVs (10). The expression of antigenically distinct VLPs and production of antisera to VLPs are needed to clarify the antigenic relationship among NLVs.

This paper describes the cDNA cloning and baculovirus expression of the viral capsid gene of the Seto virus (SeV), a member of GI NLV. In addition, we report on the development and evaluation of an antigen-detection ELISA based on the antisera to the recombinant capsid protein.

A stool specimen (124/89 in Table I) from a Seto outbreak was used to clone the capsid gene. The NLV detected in this stool was designated the Seto virus, SeV. Viral RNA was extracted from 10% stool suspension in phosphate buffered saline using Trizol™ (Gibco BRL, Gaithersburg, MD). For cDNA synthesis, oligo-dT15 (Promega Co., Madison, WI) and M-MLV reverse transcriptase (Gibco BRL) were used. A semi-nested

PCR was performed to amplify the entire ORF2 gene. The first PCR used forward primer G1F1 (5'-TGCCCGAATTCGTAAATGAT-3') (position 5343 to 5362 in Norwalk virus genome, Genbank accession no. M87661) and a reverse primer G1R0 (5'-GCCATTATCGGCGCARACCAAGCC-3') (position 6931 to 6954), and the second PCR used forward primer G1F0 (5'-GTAAATGATGATGGCGTCTAAGGA-3') (position 5354 to 5377) and G1R0. An approximately 1.6 kb PCR product was cloned into a TA cloning vector pCR2.1 (Invitrogen, San Diego, CA) to generate pCR[SeV]. Nucleotide (nt) sequence analysis of the 1.6 kb insert showed that it contained the entire ORF2 of SeV and it predicted to encode a 530 amino acids (aa) capsid protein. A comparison of the ORF2 nt sequence of SeV with those of known NLVs indicated that SeV showed the highest identity with KY89 (97%), followed by NV (89%), Chiba virus (CV) (66%), Southampton virus (62%) and Desert Shield virus (62%). SeV had a lower nt identity with GII NLVs including the Snow Mountain virus (52%), Hawaii virus (52%) and Mexico viruses (52%). The phylogenetic analysis of the ORF2 genes of SeV and representative NLVs indicated that SeV is closest to KY89 (Fig. 1).

The ORF2 gene of SeV was isolated from pCR [SeV] by digestion with *Eco* RI and inserted into a baculovirus transfer vector pVL1392 (Pharmingen, San Diego, CA) at the same *Eco* RI site (pVL[SeV]). Sf9 cells (Riken Cell Bank, Tsukuba) were cotransfected with 50 ng linearized wild-type *Autographa californica* nuclear polyhedrosis virus DNA (BaculoGold kit, Pharmingen, San Diego, CA) and 1 μg pVL[SeV] by the lipofectin-mediated method. A recombinant baculovirus, designated as Ac[SeV], was selected by two rounds of plaque purification. Tn5 cells (Invitrogen, San Diego, CA) were infected with Ac[SeV] at MOI 10, and the cells were harvested 6 days postinfection (p.i.) at 26.5℃. The expression of recombinant proteins was followed by SDS-10% polyacrylamide gel electrophoresis (16). Samples were prepared for electrophoresis by boiling for 3 min prior to loading. A major protein band with a molecular mass of 58 kDa was observed in the infected cells 2 days p.i., and the expression reached its maximum at 6 days p.i. (data