The ORF3 protein of hepatitis E virus binds to Src homology 3 domains and activates MAPK. J. Biol. Chem. **276:**42389–42400.
9. **Li, T. C., N. Takeda, and T. Miyamura.** 2001. Oral administration of hepatitis E virus-like particles induces a systemic and mucosal immune response in mice. Vaccine **19:**3476–3484.
10. **Li, T. C., Y. Suzaki, Y. Ami, T. N. Dhole, T. Miyamura, and N. Takeda.** 2004. Protection of cynomolgus monkeys against HEV infection by oral administration of recombinant hepatitis E virus-like particles. Vaccine **22:**370–377.
11. **Li, T. C., Y. Yamakawa, K. Suzuki, M. Tatsumi, M. A. Razak, T. Uchida, N. Takeda, and T. Miyamura.** 1997. Expression and self-assembly of empty virus-like particles of hepatitis E virus. J. Virol. **71:**7207–7213.
12. **Magden, J., N. Takeda, T. C. Li, P. Auvinen, T. Ahola, T. Miyamura, A. Merits, and L. Kaariainen.** 2001. Virus-specific mRNA capping enzyme encoded by hepatitis E virus. J. Virol. **75:**6249–6255.
13. **Niikura, M., S. Takamura, G. Kim, S. Kawai, M. Saijo, S. Morikawa, I. Kurane, T. C. Li, N. Takeda, and Y. Yasutomi.** 2002. Chimeric recombinant hepatitis E virus-like particles as an oral vaccine vehicle presenting foreign epitopes. Virology **293:**273–280.
14. **Olson, A. J., G. Bricogne, and S. C. Harrison.** 1983. Structure of tomato busy stunt virus. IV. The virus particle at 2.9 A resolution. J. Mol. Biol. **171:**61–93.
15. **Prasad, B. V., M. E. Hardy, T. Dokland, J. Bella, M. G. Rossmann, and M. K. Estes.** 1999. X-ray crystallographic structure of the Norwalk virus capsid. Science **286:**287–290.
16. **Purcell, R. H., and S. U. Emerson.** 2001. Hepatitis E virus, p. 3051–3061. *In* D. M. Knipe and P. M. Howley (ed.), Fields virology, 4th ed., vol. 1. Lippincott Williams & Wilkins, Philadelphia, Pa.
17. **Robinson, R. A., W. H. Burgess, S. U. Emerson, R. S. Leibowitz, S. A. Sosnovtseva, S. Tsarev, and R. H. Purcell.** 1998. Structural characterization of recombinant hepatitis E virus ORF2 proteins in baculovirus-infected insect cells. Protein Expr. Purif. **12:**75–84.
18. **Schofield, D. J., J. Glamann, S. U. Emerson, and R. H. Purcell.** 2000. Identification by phage display and characterization of two neutralizing chimpanzee monoclonal antibodies to the hepatitis E virus capsid protein. J. Virol. **74:**5548–5555.
19. **Stewart, L. M. D., and R. D. Possee.** 1993. Baculovirus expression vectors, p. 227–256. *In* A. J. Davidson and R. M. Elliotts (ed.), Molecular virology: a practical approach. IRL Press, Oxford, United Kingdom.
20. **Takamura, S., M. Niikura, T. C. Li, N. Takeda, S. Kusagawa, Y. Takebe, T. Miyamura, and Y. Yasutomi.** 2004. DNA vaccine-encapsulated virus-like particles derived from an orally transmissible virus stimulate mucosal and systemic immune responses by oral administration. Gene Ther. **11:**628–635.
21. **Tam, A. W., M. M. Smith, M. E. Guerra, C. C. Huang, D. W. Bradley, K. E. Fry, and G. R. Reyes.** 1991. Hepatitis E virus (HEV): molecular cloning and sequencing of the full-length viral genome. Virology **185:**120–131.
22. **Virus Taxonomy.** 2002. http://www.ictvdb.iacr.ac.uk/Ictv/fr-fst-g.htm.
23. **Wickham, T. J., and G. R. Nemerow.** 1993. Optimization of growth methods and recombinant protein production in BTI-Tn-5B1-4 insect cells using the baculovirus expression system. Biotechnol. Prog. **9:**25–30.
24. **Xiaofang, L., M. Zafrullah, F. Ahmad, and S. Jameel.** 2001. A C-terminal hydrophobic region is required for homo-oligomerization of the hepatitis E virus capsid (ORF2) protein. J. Biomed. Biotechnol. **1:**122–128.
25. **Xing, L., K. Kato, T. Li, N. Takeda, T. Miyamura, L. Hammar, and R. H. Cheng.** 1999. Recombinant hepatitis E capsid protein self-assembles into a dual-domain T = 1 particle presenting native virus epitopes. Virology **265:**35–45.
26. **Yarbough, P. O., A. W. Tam, K. E. Fry, K. Krawczynski, K. A. McCaustland, D. W. Bradley, and G. R. Reyes.** 1991. Hepatitis E virus: identification of type-common epitopes. J. Virol. **65:**5790–5797.
27. **Zafrullah, M., M. H. Ozdener, S. K. Panda, and S. Jameel.** 1997. The ORF3 protein of hepatitis E virus is a phosphoprotein that associates with the cytoskeleton. J. Virol. **71:**9045–9053.
28. **Zhong, Y., J. Cheng, Y. Liu, J. Dong, J. Yang, and L. Zhang.** 2000. Expression of human single-chain variable fragment antibody against non-structural protein 3 of hepatitis C virus antigen in e. coli. Zhonghua Gan Zang Bing Za Zhi **8:**171–173. (In Chinese.)

Hepatitis E Virus Transmission from Wild Boar Meat

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We investigated a case of hepatitis E acquired after persons ate wild boar meat. Genotype 3 hepatitis E virus (HEV) RNA was detected in both patient serum and wild boar meat. These findings provided direct evidence of zoonotic foodborne transmission of HEV from a wild boar to a human.

Hepatitis E virus (HEV), a causative agent of human hepatitis E, is a single-stranded positive-sense RNA virus recently classified as the sole member of the genus *Hepevirus* in the family *Hepeviridae* (1,2). HEV is transmitted primarily by the fecal-oral route through contaminated drinking water. However, recent studies have demonstrated that various animal species have serum antibodies to HEV, suggesting that hepatitis E is a zoonotic disease (3). In Japan, 4 hepatitis E cases have been linked directly to eating raw deer meat (4), and several cases of acute hepatitis E have been epidemiologically linked to eating undercooked pork liver or wild boar meat (5,6). These cases provide convincing evidence of zoonotic food-borne HEV transmission. We report direct evidence of HEV transmission from a wild boar to a human.

The Study

A 57-year-old woman came to Iizuka Hospital on March 12, 2005, with malaise and anorexia. Although she was a healthy hepatitis B virus carrier and negative for serologic markers of hepatitis A and C, testing upon admission showed elevated levels of liver enzymes (alanine aminotranferase 752 IU/L, aspartate aminotransferase 507 IU/L, and γ-glutamyl transpeptidase 225U/L). A serum sample collected on March 16 was positive for both immunoglobulin M (IgM) and IgG antibodies to HEV when tested by an antibody enzyme-linked immunosorbent assay using recombinant viruslike particles (7). This led to the diagnosis of hepatitis E. The hepatitis was typical, acute, and self-limiting, and the patient recovered by the end of March.

The patient's husband traditionally hunted boar for food 3 or 4 times a year, and she had eaten boar meat on 2 occasions. With her husband, she ate the meat as part of a hot pot on December 28, 2004, 11 weeks before her illness, and again, grilled, on January 19, 2005, along with 10 other people (including her husband) 8 weeks before her illness. Disease did not develop in the other 10 people. Except for this wild boar meat, the patient had not eaten meat or liver from other wild animals. Since she had not traveled abroad in the past 30 years, transmission must have occurred in Japan. Two portions of meat from the wild boar (meats 1 and 2) eaten on December 28, 2004, and 1 portion from the other wild boar (meat 3) eaten on January 19, 2005, remained and were frozen.

Juice was obtained from the sliced meat by centrifugation at 10,000 × *g* for 15 min. The supernatant was used for RNA extraction. A nested reverse transcription–polymerase chain reaction (RT-PCR) was conducted to amplify part of open reading frame 2 (ORF2), which corresponds to nucleotides (nt) 5939–6297 of the genotype 1 HEV genome (GenBank D10330), with external sense primer HEV-F1 (5′-TAYCGHAAYCAAGGHTGGCG-3′) and antisense primer HEV-R2 (5′-TGYTGGTTRTCRTARTCCTG-3′). A nested PCR was conducted with internal sense primer HEV-F2 (5′-GGBGTBGCNGAGGAGGAGGC-3′) and internal antisense primer HEV-R1 (5′-CGACGAAATYAATTCTGTCG-3′). This procedure allows amplification of HEV 1, 3, and 4 genotypes. A PCR product of 359 bp including the primer sequences was obtained from meat 3 by nested PCR. However, meats 1 and 2 were negative. HEV RNA was not detected in the patient's serum by the same amplification method. This may have resulted from an extremely small amount of RNA.

New primers for the nested RT-PCR were designed for a region within the 359 base region based on the meat 3 sequences, which corresponded to nt 5983–6243. The first PCR was performed with external sense primer HEV-WB-F1 (5′-ACCTCTGGCCTGGTAATGCT-3′) and antisense primer HEV-WB-R2 (5′-GAGAAGCGTATCAGCAAGGT-3′). The nested PCR was performed with internal sense primer HEV-WB-F2 (5′-TATTCATGGCTCTCCTGTCA-3′) and internal antisense primer HEV-WB-R1 (5′-ACAGTGTCAGAGTAATGCCT-3′). These primers allowed amplification of 281 nt, including the primer sequences from the patient serum collected on March 16, 2005. In contrast, meats 1 and 2 were negative with these new primers.

To further analyze the RNA in the patient serum and meat 3, RNA genomes encoding an entire ORF2 were

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amplified as overlapping segments, nucleotide sequences were determined, and phylogenetic analysis was carried out with avian HEV as an outgroup. Avian HEV is a causative agent of chicken hepatitis-splenomegaly syndrome (8). Two sequences, 1 from the patient (DQ079629) and the other from meat 3 (DQ079630), were classified into genotype 3 (Figure). Only 1 nt difference was observed in the 1,980 nt of the entire ORF2; the nucleotide sequence identity was 99.95%. The difference was not accompanied by any amino acid changes. These data demonstrated that HEV infection was transmitted from the wild boar meat to the patient on January 19, 2005.

Conclusions

Currently, deer, pig, and wild boar are suspected sources of foodborne zoonotic transmission of HEV in Japan, and genotypes 3 and 4 of HEV are believed to be indigenous (4–6,9,10). Direct evidence for transmission of genotype 3 HEV from animals to humans was observed in acute hepatitis in 4 persons who had eaten uncooked deer meat that contained $\approx 10^7$ copies of HEV RNA (4). However, the rare finding of HEV antibody-positive deer in Japan suggest that deer are not the major zoonotic reservoir of HEV in this country (11). In contrast, high antibody-positive rates in domestic pig and wild boar, including HEV genotypes 3 and 4, have been frequently detected, suggesting that persons who eat uncooked meat are at risk for infection with HEV (12,13). This report is the first to provide direct evidence of zoonotic foodborne genotype 3 HEV transmission from wild boar to a human.

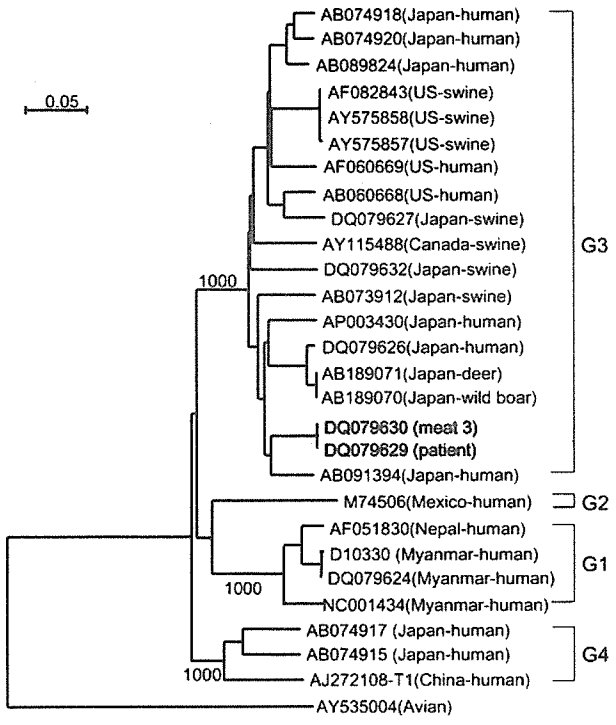

Figure. Phylogenetic tree of hepatitis E virus (HEV) reconstructed with avian HEV as an outgroup. Nucleotide sequences of the entire open reading frame 2 were analyzed by the neighbor-joining method. The bootstrap values correspond to 1,000 replications. The 2 nucleotide sequences characterized in this study are shown in **bold**. The horizontal scale bar at the top left indicates nucleotide substitutions per site.

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References

1. Purcell RH, Emerson SU. Hepatitis E virus. In: Knipe DM, Howley PM, editors. Fields virology. Fourth ed. Philadelphia: Lippincott Williams & Wilkins; 2001. p. 3051–61.
2. Emerson SU, Anderson D, Arankalle A, Meng XJ, Purdy M, Schlauder GG, et al. Hepevirus. In: Fauquet CM, Mayo MA, Maniloff J, Desselberger U, Ball LA, editors. Virus taxonomy: eighth report of the ICTV. London: Elsevier/Academic Press; 2004. p. 851–5.
3. Meng X-J. Zoonotic and xenozoonotic risks of the hepatitis E virus. Infect Dis Rev. 2000;2:35–41.
4. Tei S, Kitajima N, Takahashi K, Mishiro S. Zoonotic transmission of hepatitis E virus from deer to human beings. Lancet. 2003;362:371–3.
5. Yazaki Y, Mizuo H, Takahashi M, Nishizawa T, Sasaki N, Gotanda Y, et al. Sporadic acute or fulminant hepatitis E in Hokkaido, Japan, may be food-borne, as suggested by the presence of hepatitis E virus in pig liver as food. J Gen Virol. 2003;84:2351–7.
6. Matsuda H, Okada K, Takahashi K, Mishiro S. Severe hepatitis E virus infection after ingestion of uncooked liver from a wild boar. J Infect Dis. 2003;188:944.
7. Li TC, Yamakawa Y, Suzuki K, Tatsumi M, Razak MA, Uchida T, et al. Expression and self-assembly of empty virus-like particles of hepatitis E virus. J Virol. 1997;71:7207–13.
8. Haqshenas G, Shivaprasad HL, Woolcock PR, Read DH, Meng XJ. Genetic identification and characterization of a novel virus related to human hepatitis E virus from chickens with hepatitis-splenomegaly syndrome in the United States. J Gen Virol. 2001;82:2449–62.
9. Tamada Y, Yano K, Yatsuhashi H, Inoue O, Mawatari F, Ishibashi H. Consumption of wild boar linked to cases of hepatitis E. J Hepatol. 2004;40:869–70.
10. Masuda JI, Yano K, Tamada Y, Takii Y, Ito M, Omagari K, et al. Acute hepatitis E of a man who consumed wild boar meat prior to the onset of illness in Nagasaki, Japan. Hepatol Res. 2005;31:178–83.
11. Sonoda H, Abe M, Sugimoto T, Sato Y, Bando M, Fukui E, et al. Prevalence of hepatitis E virus (HEV) infection in wild boars and deer and genetic identification of a genotype 3 HEV from a boar in Japan. J Clin Microbiol. 2004;42:5371–4.
12. Meng XJ, Halbur PG, Shapiro MS, Govindarajan S, Bruna JD, Mushahwar IK, et al. Genetic and experimental evidence for cross-species infection by swine hepatitis E virus. J Virol. 1998;72:9714–21.

13. Takahashi M, Nishizawa T, Miyajima H, Gotanda Y, Iita T, Tsuda F, et al. Swine hepatitis E virus strains in Japan form four phylogenetic clusters comparable with those of Japanese isolates of human hepatitis E virus. J Gen Virol. 2003;84:851–62.

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Expression and Antigenicity of Virus-Like Particles of Norovirus and Their Application for Detection of Noroviruses in Stool Samples

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Human noroviruses (NoVs), members of the genus *Norovirus* in the family *Caliciviridae*, are the leading agents of nonbacterial acute gastroenteritis worldwide. Human NoVs are currently divided into at least two genogroups, genogroup I (GI) and genogroup II (GII), each of which contains at least 14 and 17 genotypes. To explore the genetic and antigenic relationship among NoVs, we expressed the capsid protein of four genetically distinct NoVs, the GI/3 Kashiwa645 virus, the GII/3 Sanbu809 virus, the GII/5 Ichikawa754 virus, and the GII/7 Osaka10-25 virus in baculovirus expression system. An antigen enzyme-linked immunosorbent assay (ELISA) with hyperimmune serum against the four recombinant capsid proteins and characterized previously three capsid proteins derived from GI/1, GI/4, and GII/12 was developed to detect the NoVs antigen in stools. The antigen ELISA was highly specific to the homotypic strains, allowing assignment of a strain to a Norovirus genetic cluster within a genogroup. ***J. Med. Virol. 76:129–136, 2005.*** © 2005 Wiley-Liss, Inc.

KEY WORDS: norovirus; ELISA; gastroenteritis; virus-like particle; calicivirus

INTRODUCTION

Norovirus (NoV), a member of one of four genera in the family *Caliciviridae* [Atmar and Estes, 2001; Green et al., 2001a], is a major cause of water and food-borne acute nonbacterial gastroenteritis, and is composed of many genetically distinct viruses. [Kapikian, 1994; Kapikian et al., 1996; Estes et al., 1997]. The detection and molecular characterization of NoV have been hampered due to the lack of cell culture and small animal models [Duizer et al., 2004]. However, recent progress in molecular cloning and the sequence determination of RNA-dependent RNA polymerase and capsid protein genes of the NoVs has enabled us to classify NoVs into at least two genogroups: genogroup I (GI) and genogroup II (GII) [Green et al., 2001b]. In a previous study, a scheme for genotyping based on the N-terminal capsid protein was demonstrated [Katayama et al., 2002], and a recent report proposed that GI and GII contain at least 14 and 17 genotypes, respectively [Kageyama et al., 2004].

The NoV contains a single-stranded positive-sense RNA genome of 7.6 kb excluding the poly-A tail that encodes three open reading frames (ORFs) [Jiang et al., 1993; Lambden et al., 1993]. ORF1 encodes a nonstructural polyprotein, which is cleaved into functional proteins by a virus-encoded 3C-like protease, and ORF2 and ORF3 encode the major capsid protein VP1 and minor capsid protein VP2, respectively [Jiang et al., 1992; Glass et al., 2000]. When the ORF2 gene alone or the 3′ end of 2.3 kb, including ORF2, ORF3, and the 3′ noncoding region is expressed by a recombinant

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baculovirus, the recombinant protein spontaneously self-assembles into virus-like particles (VLPs) which are antigenically and morphologically similar to the native virion [Jiang et al., 1992, 2002; Lew et al., 1994a,b; Dingle et al., 1995; Hale et al., 1999; Kobayashi et al., 2000a,b,c; Belliot et al., 2001]. The VLPs have been used successfully for in structural studies [Prasad et al., 1994, 1999], as well as the development of enzyme-linked immunosorbent assay (ELISA) for serological diagnosis of NoV infection [Gray et al., 1993; Green et al., 1993; Parker et al., 1993, 1994, 1995]. Though antigen ELISA using hyperimmune antisera raised against the VLPs has been developed to detect NoV in stools [Graham et al., 1994; Jiang et al., 1995a,b,c; Hale et al., 1999], the sensitivity is low due to the ability of the ELISA to detect only strains closely related to one used to produce the hyperimmune serum [Numata et al., 1994; Jiang et al., 1995a,b,c]. The expression of antigenically distinct more VLPs and the preparation of antisera to them are needed to clarify the antigenic relationship among NoVs.

The expression of four capsid proteins from the GI/3, GII/3, GII/5 and GII/7 NoVs and the preparation of the VLPs are described and the antigenic relationship among seven VLPs, including three NoV VPLs from GI/1, G1/4, and GII/12 prepared previously [Kobayashi et al., 2000a,b,c], and the detection of NoVs in fecal specimens by using an ELISA are described.

MATERIALS AND METHODS

Viruses, RT-PCR, and molecular cloning. Hu/NV/GI/Kashiwa645/1999/JP (Kashiwa645, sequence accession number BD011871), Hu/NV/GII/Sanbu809/1998/JP (Sanbu809, BD011876), and Hu/NV/GII/Ichikawa745/1998/JP (Ichikawa745, BD011877) were associated with outbreaks of acute gastroenteritis as reported by the Kashiwa, Sanbu, and Ichikawa Health Centers in Chiba prefecture, Japan in 1989–1999. Hu/NV/GII/Osaka10-25/1999/JP (Osaka10-25, BD011881) was associated with an outbreak of acute gastroenteritis in Osaka Prefecture, Japan, in 1999. Stool samples containing these viruses were homogenized in phosphate buffered saline (PBS), and a 10% suspension was prepared. After centrifugation at 3,000*g* for 10min, the supernatant was used for RNA extraction with Trizol™ (Gibco BRL, Gaithersburg, MD) [Kobayashi et al., 2000a]. The cDNA synthesis was performed with an oligo-dT15 (Promega Co., Madison, WI) and reverse transcriptase from the Molony murine leukemia virus (Gibco BRL) as described by Green et al. [1997]. An approximately 1.6 kb fragment that encodes the entire VP1 of Kashiwa645, Sanbu809 and Ichikawa745, and a 2.3 kb fragment that encodes the VP1, VP2, 3′ noncoding region, and poly-A of Osaka10-25 were amplified with the primers shown in Table I. The PCR was performed in 100 μl of the reaction mixture containing 2.5 U of Takara Ex Taq (TaKaRa Shuzo Co., Ltd., Kyoto), 10 μl of 10 × PCR buffer, 8 μl of 25 mM dNTPs, 1 μl of 50 μM of each primer, and 5 μl of cDNA. After an initial denaturation at 94°C for 5 min, 35 cycles of amplification were performed using the GeneAmp PCR System 9600 (PE Biosystems, Foster City, CA). 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. The amplified fragments were cloned into a pCR2.1 plasmid (Invitrogen, San Diego, CA). The nucleotide sequences were determined with an ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Foster City, CA) and phylogenetically analyzed as described previously [Katayama et al., 2002; Kageyama et al., 2004].

Recombinant VLPs

The amplified fragment was isolated from the vector by digestion with the appropriate restriction endonucleases, and inserted into a baculovirus transfer vector pVL1392 (Pharmingen, San Diego, CA), which was used to cotransfect the Sf9 cells (Riken Cell Bank, Tsukuba) with linearized wild-type *Autographa californica* nuclear polyhedrosis virus DNA (Pharmingen) by the lipofectin-mediated method, as described by the manufacturer (Invitrogen). Recombinant baculoviruses thus obtained were selected by two rounds of plaque purification and used to prepare the seed viruses. Tn5 cells (Invitrogen) were infected with the seed virus at a multiplicity of infection (m.o.i.) of 10, incubated at 26.5°C, and the culture medium was harvested at 5–6 days post infection (p.i.). Leupeptin 10 μM (Sigma Chemicals, St. Louis, MO) and 2 μM pepstatin A (Sigma Chemicals) were added to the medium at 3 days p.i. The expression of the recombinant protein in the medium was monitored by sodium dodecyl sulfate-10% polyacrylamide gel electrophoresis (SDS–PAGE) followed by staining with Coomassie brilliant blue. The culture medium was clarified by centrifugation at 10,000*g* for 30 min, and then the VLPs in the supernatant were concentrated by centrifugation at 100,000*g* for 4 hr in a Beckman SW27 rotor. The pellet was resuspended in Grace's medium (DIFCO, Franklin Lakes, New Jersey) and examined by electron microscopy (EM). The VLPs were further purified by $CsCl_2$ equilibrium gradient density gradient centrifugation at 100,000*g* for 24 hr at

TABLE I. Primer Sequences

NoV	Forward primer	Reverse primer
Kashiwa 645	G1/F2 (5′-AATGATGATGGCGTCTAAAGGA-3′)	707R1 (5′-TGAGCCATTATGATCTTCTGATGC-3′)
Sanbu 809	G2/F3 (5′-TTGTGAATGAAGATGGCGTCGA-3′)	MVR1 (5′-AATTATTGAATCCTTCTACGCCCG-3′)
Ichikawa 754	G2/F3 (5′-TTGTGAATGAAGATGGCGTCGA-3′)	SMVR1 (5′-AATTACTGAACCCTTCTACGCCCATTTC-3′)
Osaka 10–25	G2FCR7 (5′-ATGAAGATGGCGTCGAATGACG-3′)	Oligo-dT(33)

16°C. The purified VLPs were used to immunize the animals.

Hyperimmune Sera

Hyperimmune sera to recombinant Kashiwa645 VLPs (r645), Sanbu809 VLPs (r809), Ichikawa754 VLPs (r754), and Osaka10-25 VLPs (r10-25) were prepared in rabbits. The first subcutaneous injection was performed with the purified 500 μg VLPs in Freund's complete adjuvant. After 3 weeks, the animals received two or three booster injections of 250 μg of the VLPs in Freund's incomplete adjuvant at intervals of 1 week. The animals were bled 1 week after the last booster injection. The antibody titers of rabbit hyperimmune sera to VLPs were tested in parallel by an indirect ELISA, as described previously for rSeto 124 VLPs [Kobayashi et al., 2000b] except that a VLP concentration of 0.5 μg/ml was used to coat the ELISA plate. ELISA titers were expressed as the reciprocal of the highest dilution of antiserum giving an optical density (OD) at 450 nm of >0.2.

Antigen ELISA

An antigen detection ELISA was developed using the rabbit hyperimmune sera to four recombinant capsid proteins (r645, r809, r754, and r10–25), and three previously characterized VLPs, Seto 124 VLPs (rSeto) [Kobayashi et al., 2000b], Chiba 407 VLPs (rChiba) [Kobayashi et al., 2000a], and Chitta 1876 VLPs (rChitta) [Kobayashi et al., 2000c]. Microtiter plates (96-well) (Maxisorp, Nunc, Denmark) were coated with 100 μl (0.5 μg of IgG/ml) of the rabbit preimmune (1:8,800 dilution) or hyperimmune sera (1:8,800–12,000 dilutions) in a coating buffer (0.05M carbonate-bicarbonate buffer, pH 9.6) overnight at 4°C. The well was washed twice with PBS containing Tween 20 (PBS-T), and then blocked with 0.5% bovine serum albumin in PBS overnight at 4°C. One-hundred microliter of a 10% stool sample was added to the well and incubated 1 hr at room temperature. After washing the well four times with PBS-T, 100 μl of peroxidase-conjugated rabbit antiserum to VLPs were added to the well and incubated for 1 hr at room temperature. The microplate was washed four times with PBS-T, and then 100 μl of substrate, tetrametyl bentijin (TMB), was added. The plate was left for 30 min at room temperature, and the reaction was stopped with 100 μl of 0.6N H_2SO_4. The OD_{450} value of the reactions at both the hyperimmune and preimmune sera was measured. The sample was considered positive when the difference between the OD_{450} values for the hyperimmune and preimmune sera was >0.15 and the ratio was >2 [Kobayashi et al., 2000c].

Detection of NoV in Stool Specimens by RT-PCR

Extraction of viral RNA from the stools and cDNA synthesis were performed as described above. A forward primer G1F1 and a reverse primer G1R1 were used to amplify the N-terminal VP1 of GI NoV, and a forward primer G2F1 and a reverse primer G2R1 were used to amplify the same region of the GII NoV as previously described [Kobayashi et al., 2000c; Kojima et al., 2002]. The reaction was carried out in 50 μl of the solution containing 1.25 U of Ex Taq polymerase (TaKaRa), 5 μl of 10 × PCR buffer (100 mM Tris-HCl, 15 mM $MgCl_2$, 500 mM KCl), 5 μl of 25 mM deoxynucleotide mixture, 0.5 μM of each primer, and 2 μl of cDNA. After an initial denaturation at 94°C for 5 min, 35 cycles of amplification were performed using the GeneAmp PCR System 9600 (PE Biosystems). The nucleotide sequence and phylogenetic analyses were performed as described above.

Phylogenetic Analysis

Nucleotide sequences of the entire VP1 capsid protein and N-terminal VP1 were aligned with Clustal X (http://www-igbmc.u-strasbg.fr/BioInfo/). The genetic distances were calculated by Kimura's two parameter method [Kimura, 1980], and a distance matrix file was created as described previously [Katayama et al., 2002]. The phylogenetic dendrogram was constructed by the neighbor-joining method [Saitou and Nei, 1987] with 1,000 times of bootstrap resampling [Feisenstein, 1985] as described previously [Katayama et al., 2002].

Genome Sequences

The GenBank accession numbers of the entire VP1 sequences of the strains used in this study are as follows: Aichi124-89 (Seto), accession no. AB031013; Alphatron, AF195847; Amsterdam, AF195848; Appalachicola, AF414406; Arg320, AF190817; Auckland, U46039; M7, AY130761; Birmingham, AJ277612; Boxer, AF538679; Bristol, X76716; BS5, AF093797; Burwash Landing, AF414425; Camberwell, AF145896; Chiba, AB022679; Chitta, AB032758; Desert Shield, U04469; Dijon, AF472623; Erfurt, AF427118; Florida, AF414407; Girlington, AJ277606; Grimsby, AJ004864; Gwynedd, AF414408; Hawaii, U07611; Hillingdon, AJ277607; Honolulu, AF414403; Hesse, AF414406, IdahoFalls, AY054299; Kashiwa47, AB078334; LittleRock; AF414405; Leeds, AJ277608; Manchester, X86560; Mexico, U22498; Melksham, X81879; Miami, AF414410; Musgrove, AJ277614; NewOrleans, AF414422; Norwalk/68, M87661; QueenArms, AJ313030; SaintCloud, AF414427; SaitamaU1, AB039775; SaitamaU16, AB067539; SaitamaU25, AB067543; Saitama SzUG1; AB039774, Seacroft, AJ277620; Sindlesham, AJ277615; Snow Mountain , U70059; Southampton, L07418; Stavanger, AF145709; Toronto, U02030; Valetta, AJ277616; Virginia, AY038599; White River, AF414423; WhiteRose, AJ277610, Winchester, AJ277609; Wortley, AJ277618; Mc37, AY237415, WUG1, AB081723, Kashiwa 645, BD011871, Sanbu 809, BD011876, Ichikawa 754, BD011877, and Osaka 10-25, BD011881.

RESULTS

Characterization of Four NoV Strains

To classify genetically the four NoVs, the entire VP1 genes were amplified by RT-PCR and the nucleotide

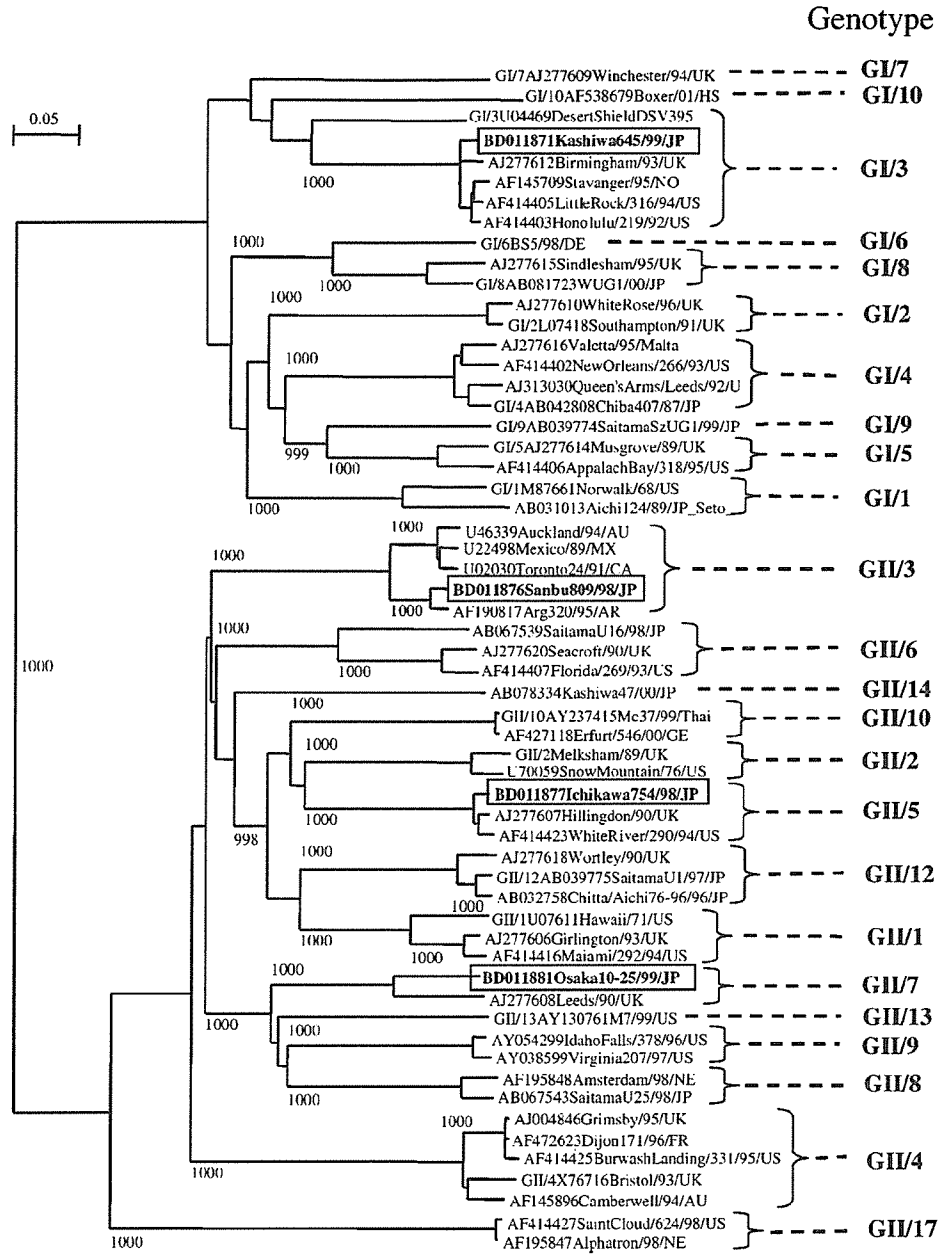

Fig. 1. Phylogenetic tree of NoVs based on entire VP1 nucleotide sequences. The numbers on each branch indicate the bootstrap values for the clusters supported by each branch. Cryptograms indicating the accession number/location or strain name/(isolate)/year/country are given for the strains. Putative genotypes are indicated for each cluster. The names GI/1 to GI/10, and GII/1 to GII/17, with the exception of GII/11, GII/15, and GII/16, are from our previous study [Katayama et al., 2002]. The complete sequences of VP1 of GII/11, GII/15, and GII/16 are not available. The four NoVs characterized in this study are shown in boldface and boxed.

sequence was determined. The phylogenetic analysis of these viruses and representative NoVs is shown in Fig. 1. Kashiwa 645 was close to Birmingham/93/UK and classified into GI/3 where Desert Shield/90/US is the representative strain. The other three strains had higher nucleotide sequence identities to GII than to the GI NoVs. Sanbu 809 was classified into GII/3 with the Toronto/93/CA and Mexico/89/MX viruses. Ichikawa 754 belongs to the GII/5 Hillingdon/90/UK genotype, whereas Osaka 10-25 was classified into GII/7 with the Leeds/90/UK virus.

Expression of the Capsid Proteins in Insect Cells

Tn5 cells (Tn5) were infected with the virus at m.o.i. 10, and the cells were incubated at 26.5°C [Li et al., 1997; Kobayashi et al., 2000a,b,c]. The expressed recombinant proteins were analyzed by 10% SDS–PAGE. A major

protein band with a molecular mass of 58–60 kDa was observed in the infected cells 2 days p.i., and the expression reached to a maximum 6 days p.i. The size of the proteins were in agreement with the molecular mass calculated from the 545, 548, 540, and 541 amino acids of Kashiwa 645, Sanbu 809, Ichikawa 754, and the Osaka 10–25 capsid proteins, respectively. The supernatant was collected at 6 days p.i., centrifuged at 100,000*g* for 2 hr in a Beckman TLA-45 rotor, and then the pellet was examined by EM. Uniform, round-shaped empty VLPs with a 38 nm diameter were observed at over 100 particles per EM field at a magnification of 20,000× (data not shown).

Antigenic Relationships of Newly Expressed Four VLPs With Previously Characterized Three VLPs

Rabbit hyperimmune antisera raised against purified r645, r809, r754, and r10–25 had titers as high as 1:409,600–1:819,200. The hyperimmune serum was not adsorbed with the wild baculovirus-infected Tn5 cell lysate, because the OD values in the antibody ELISA were negligible even when 0.5 μg protein/ml of the lysate was used to coat the microplate wells (data not shown).

The antigenic relationship of the four NoV strains was examined with three previously characterized VLPs (Table II). The highest antibody titers were detected in hyperimmune sera against homologous recombinant capsid antigens for all seven strains. Although variable cross-reactivity was detected among different recombinant antigens, higher cross-reactivity was observed with the intra-genogroup strains than with the inter-genogroup strains. For example, GI Kashiwa 645 is genetically closer to two GI NoVs, Seto 124, and Chiba 407, than the other four GII NoVs. The anti-r645 hyperimmune serum had higher antibody titers to rSeto and rChiba than to the other four GII VLPs. The hyperimmune sera to rSeto and rChiba also had higher antibody titers to the other two GI VLPs than to the other four GII VLPs. Conversely, the four GII NoVs were genetically closer to each other than to the three GI NoVs, and each GII hyperimmune serum had a higher antibody titer to the other three GII VLPs. Thus, NoVs in the same genogroup were not only genetically but also antigenically closer to one another than to those in the different genogroup.

To test further the specificity of hyperimmune serum to seven recombinant capsid proteins, an antigen ELISA was developed. In this experiment, hyperimmune sera (50 ng/well) were used to coat microplate wells to capture the recombinant VLPs. As shown in Table III, the OD values in the homologous reaction decreased in a dose-dependent manner. Little cross-reactions between different genotypes were observed. In contrast, high sensitivity was found in the homologous reactions although the limit of detection is 0.4 ng/ml as observed in the reaction with rChiba and r809, and this corresponds to 2.5×10^6 particles of NoVs.

Detection of NoV Antigen in Stool Specimens

To test the performance of the antigen ELISA, NoVs detection was carried out with stool specimens from acute gastroenteritis patients. Microplates were coated with the rabbit preimmune or hyperimmune sera to capture the antigen in the stool specimens, and peroxidase-conjugated antiserum was used as the detector antibody. In control experiments, antisera against recombinant VLPs efficiently captured at least 4 ng of the homologous antigen but not the heterologous antigen (Table III). The preimmune sera did not capture any of the VLPs at any concentration (the OD value was usually less than 0.05). Two to three representative stool specimens were selected from outbreaks associated with the GI/1, GI/3, GI/4, GII/3, GII/5, GII/7, and GII/12 genotypes to evaluate the antigen ELISA. All specimens were positive by RT-PCR targeting the N-terminal capsid region, and the amplified fragments were genotyped by sequencing analyses followed by phylogenetic analyses (Table IV). All hyperimmune sera except GI/1 Seto reacted only to the homologous genotype samples. In Seto virus detection, all three GI/3 and one of the three GI/4 samples were positive by ELISA. Interestingly, stool samples 98-MC4 (GI/1) and 2000K-518 (GI/3) were negative by EM but positive by the ELISA, suggesting that the antigen ELISA established in this study is capable of detecting disrupted NoV

TABLE II. ELISA Titers of Seven Hyperimmune Antisera Against VLPs

VLPs[a]		Hyperimmune sera against VLP antigens						
		GI/1 Seto	GI/3 645	GI/4 Chiba	GII/3 809	GII/5 754	GII/7 10–25	GII/12 Chitta
GI/1	rSeto	**819200**[b]	25600	12800	200	12800	1600	3200
GI/3	r645	102400	**819200**	25600	400	12800	6400	6400
GI/4	rChiba	102400	25600	**819200**	800	6400	6400	6400
GII/3	r809	25600	6400	6400	**819200**	51200	51200	25600
GII/5	r754	25600	6400	3200	25600	**819200**	25600	51200
GII/7	r10-25	12800	12800	6400	25600	51200	**819200**	25600
GII/12	rChitta	25600	1600	1600	25600	51200	25600	**409600**

[a]Four VLPs: r645, r809, r754, and r10-25 and their hyperimmune sera were prepared in this study, and three VLPs: rSeto, rChiba, and rChitta and their hyperimmune sera were prepared in our previous studies[Kobayashi et al., 2000a,b,c].
[b]ELISA titers were expressed as the reciprocal of the highest dilution of antiserum giving an optical density (OD) at 450 nm of >0.2. Homologous titers are shown in boldface.

TABLE III. Reactivity Between VLPs and Hyperimmune Antisera as Determined by Antigen ELISA

VLPs (ng/mL)		Hyperimmune sera						
		GI/1 Seto	GI/3 645	GI/4 Chiba	GII/3 809	GII/5 754	GII/7 10–25	GII/12 Chitta
rSeto	40	**4.007**	0.075	0.063	0.011	0.010	0.012	0.011
	4	**1.430**	0.034	0.023	0.009	0.011	0.014	0.010
	0.4	0.192	0.028	0.023	0.009	0.010	0.013	0.011
	0	0.010	0.028	0.013	0.008	0.011	0.012	0.011
r645	40	0.173	**3.235**	0.047	0.009	0.014	0.010	0.020
	4	0.061	**0.893**	0.035	0.010	0.010	0.009	0.013
	0.4	0.023	0.114	0.010	0.009	0.011	0.010	0.013
	0	0.010	0.030	0.010	0.009	0.014	0.010	0.015
r Chiba	40	0.105	0.034	**4.259**	0.009	0.012	0.010	0.011
	4	0.048	0.029	**1.408**	0.010	0.011	0.013	0.010
	0.4	0.011	0.026	**0.209**	0.010	0.010	0.010	0.010
	0	0.011	0.026	0.026	0.009	0.011	0.010	0.010
r809	40	0.060	0.028	0.023	**3.650**	0.075	0.056	0.066
	4	0.018	0.031	0.010	**1.271**	0.029	0.023	0.024
	0.4	0.013	0.034	0.007	**0.209**	0.015	0.013	0.014
	0	0.012	0.032	0.010	0.008	0.012	0.010	0.016
r754	40	0.011	0.030	0.025	0.014	**3.994**	0.041	0.083
	4	0.010	0.028	0.024	0.010	**1.329**	0.020	0.039
	0.4	0.010	0.029	0.025	0.010	0.184	0.012	0.015
	0	0.011	0.031	0.025	0.009	0.011	0.010	0.016
r10–25	40	0.018	0.049	0.025	0.016	0.107	**4.326**	0.100
	4	0.015	0.048	0.027	0.011	0.049	**1.343**	0.058
	0.4	0.010	0.038	0.025	0.010	0.017	0.160	0.021
	0	0.010	0.037	0.027	0.010	0.010	0.011	0.010
r Chitta	40	0.014	0.034	0.030	0.011	0.061	0.019	**3.356**
	4	0.011	0.031	0.026	0.010	0.022	0.012	**0.819**
	0.4	0.013	0.032	0.025	0.009	0.013	0.012	0.124
	0	0.014	0.024	0.024	0.009	0.012	0.011	0.025

The reaction was considered to be positive when the difference between the OD_{450} values for the hyperimmune and preimmune sera was >0.15 and the ratio was >2. Positive reactions were shown in boldface.

TABLE IV. Detection of NoV Antigens in Fecal Specimens by Antigen ELISA

Fecal Samples	Hyperimmune sera									
	GI/1 Seto	GI/3 645	GI/4 Chiba	GII/3 809	GII/5 754	GII/7 10–25	GII/12 Chitta	EM	RT-PCR	Genotype
2000K-600	**1.921**[a]	0.052	0.028	0.015	0.013	0.016	0.014	N/A[b]	+	GI/1
98-MC3	**1.407**	0.155	0.060	0.023	0.017	0.018	0.018	+	+	GI/1
98-MC4	**0.596**	0.086	0.056	0.029	0.017	0.018	0.017	–	+	GI/1
2000K-514	**0.498**	**1.521**	0.035	0.029	0.015	0.017	0.017	+	+	GI/3
2000K-518	**0.225**	**0.597**	0.033	0.017	0.012	0.015	0.016	–	+	GI/3
Se1	**0.450**	**2.641**	0.029	0.016	0.012	0.015	0.015	N/A	+	GI/3
2000K-691	0.038	0.040	**0.446**	0.017	0.013	0.015	0.015	+	+	GI/4
2000K-694	**0.358**	0.080	**4.899**	0.049	0.016	0.016	0.017	+	+	GI/4
96-844	0.166	0.071	**2.171**	0.017	0.014	0.015	0.017	+	+	GI/4
98K-826	0.022	0.051	0.065	**1.369**	0.016	0.039	0.066	+	+	GII/3
98K-836	0.018	0.037	0.032	**1.296**	0.015	0.042	0.030	+	+	GII/3
98-249	0.016	0.063	0.030	**1.083**	0.020	0.043	0.034	+	+	GII/3
95-277	0.016	0.034	0.023	0.015	**1.696**	0.024	0.029	+	+	GII/5
00-683	0.011	0.036	0.031	0.090	**0.428**	0.012	0.018	+	+	GII/5
00-684	0.012	0.025	0.015	0.039	**2.994**	0.041	0.136	+	+	GII/5
S99-75	0.037	0.041	0.071	0.101	0.147	**0.426**	0.168	N/A	+	GII/7
S99-21	0.008	0.021	0.035	0.021	0.082	**1.712**	0.010	N/A	+	GII/7
98-41	0.021	0.035	0.031	0.023	0.013	0.028	**1.433**	+	+	GII/12
98-2345	0.019	0.037	0.026	0.020	0.013	0.023	**0.453**	+	+	GII/12
99-1007	0.021	0.055	0.036	0.130	0.014	0.031	**2.722**	+	+	GII/12

[a]The reaction was considered to be positive when the difference between the OD_{450} values for the hyperimmune and preimmune sera was >0.15 and the ratio was >2. Positive reactions were shown in boldface.
[b]Not applicable.

antigen, although the titers were relatively low even in the homologous reaction. Another possible (even more likely) explanation is that the antigen ELISA is more sensitive than EM as described previously [Graham et al., 1994].

DISCUSSION

This study describes the cloning, sequencing, and expression of the capsid proteins of four currently circulating NoV strains and their genetic and antigenic relationships with three previously characterized strains. The phylogenetic analyses indicated that these seven strains belong to GI/1, GI/3, GI/4, GII/3, GII/5, GII/7, and GII/12. In contrast to the rapid accumulation of information for genetic diversity, at least 14 in the GI and 17 in the GII genotypes, studies on the determination of antigenic type have been relatively slow [Kageyama et al., 2004]. This is because generation of the VLPs antigen is not an easy task, and a comparative study with a panel of a large number of VLPs antigens and their antisera is difficult to be performed at the moment. Continued preparation and characterization of more VLPs antigens and their hyperimmune sera are highly useful to refine the panel.

Although the seven antigenic types were distinguishable by the hyperimmune antisera generated against the VLPs, low levels of cross-reaction were observed among these strains when the antibody ELISA was used. Because no NoV specific antibody was detected in the preimmune animal sera, the cross-reactive antibodies in the hyperimmune antisera originated from common antigenic epitopes among NoVs. In fact, Kitamoto et al. [2002] obtained not only genogroup-specific, but also common cross-reactive monoclonal antibodies (MAbs) for four GI and six GII VLPs. Further characterization of antigenic epitopes using MAbs for the antigenic type-specific diagnosis of NoV is needed. In antibody ELISA (Table II), higher responses were observed between the intra-genogroup strains than between the inter-genogroup strains. This observation is worth noting, because it indicates that GI and GII are not only genetically, but also antigenically distinct.

Previous observations of the immune responses of patients involved in outbreaks have also explained the common antigenic epitopes. Immune responses to multiple antigenic types, most of which were caused by a single NoV strain, are often detected in outbreaks of gastroenteritis [Vipond et al., 2004]. Higher responses to homotypic than to heterotypic strains may allow us to make a seroresponse-based diagnosis of NoV infection. Indeed, we recently identified a GII/4 infection in a hospital based on the immune responses of the patients (data not shown).

As has been shown in the case of the Norwalk virus, Mexico virus, Grimsby virus, Seto virus, Chiba virus, and Chitta virus, the antigen ELISA for NoV was highly genotype-specific [Numata et al., 1994; Hale et al., 1996, 1999; Kobayashi et al., 2000a,b,c]. Further generation of VLPs antigens and hyperimmune sera against each genotype VLPs and the subsequent development of both antibody and antigen ELISAs are necessary for the detection of NoVs, for the antigenic relationship among NoVs, and for the classification of NoVs.

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REFERENCES

Atmar RL, Estes MK. 2001. Diagnosis of noncultivatable gastroenteritis viruses, the human caliciviruses. Clin Microbiol Rev 14: 15–37.

Belliot G, Noel JS, Li JF, Seto Y, Humphrey CD, Ando T, Glass RI, Monroe SS. 2001. Characterization of capsid genes, expressed in the baculovirus system, of three new genetically distinct strains of "Norwalk-like viruses." J Clin Microbiol 39:4288–4295.

Dingle KE, Lambden PR, Caul EO, Clarke IN. 1995. Human enteric Caliciviridae: The complete genome sequence and expression of virus-like particles from a genetic group II small round structured virus. J Gen Virol 76:2349–2355.

Duizer E, Schwab KJ, Neill FH, Atmar RL, Koopmans MP, Estes MK. 2004. Laboratory efforts to cultivate noroviruses. J Gen Virol 85:79–87.

Estes MK, Atmar RL, Hardy ME. 1997. Norwalk and related diarrhea viruses. In: Richmann DD, Whitley RJ, Hayden FG, editors. Clinical virology. New York: Churchill Livingstone, Inc. pp 1073–1095.

Feisenstein J. 1985. Confidence limits on phylogenies: An approach using the bootstrap. Evolution 39:783–791.

Glass PJ, White LJ, Ball JM, Leparc-Goffart I, Hardy ME, Estes MK. 2000. Norwalk virus open reading frame 3 encodes a minor structural protein. J Virol 74:6581–6591.

Graham DY, Jiang X, Tanaka T, Opekun AR, Madore HP, Estes MK. 1994. Norwalk virus infection of volunteers: New insights based on improved assays. J Infect Dis 170:34–43.

Gray JJ, Jiang X, Morgan-Capner P, Desselberger U, Estes MK. 1993. Prevalence of antibodies to Norwalk virus in England: Detection by enzyme-linked immunosorbent assay using baculovirus-expressed Norwalk virus capsid antigen. J Clin Microbiol 31:1022–1025.

Green KY, Lew JF, Jiang X, Kapikian AZ, Estes MK. 1993. Comparison of the reactivities of baculovirus-expressed recombinant Norwalk virus capsid antigen with those of the native Norwalk virus antigen in serologic assays and some epidemiologic observations. J Clin Microbiol 31:2185–2191.

Green SM, Lambden PR, Caul EO, Clarke IN. 1997. Capsid sequence diversity in small round structured viruses from recent UK outbreaks of gastroenteritis. J Med Virol 52:14–19.

Green KY, Ando T, Balayan MS, Clarke IN, Estes MK, Matson DO, Nakata S, Neill JD, Struddert MJ, J TH. 2001a. Caliciviridae. In: van Regenmortel MHV, Fauquet CM, Bishop DHL, Carsten EB, Estes MK, Lemon SM, Maniloff J, Maya MA, McGeoch DJ, Pringle CR, Wickner RB, editors. Virus taxonomy. 3rd edn, San Diego: Academic Press, Inc. pp 725–734.

Green KY, Chanock RM, Kapikian AZ. 2001b. Human caliciviruses. In: Knipe DM, Howley PM, Griffin DE, editors. Fields virology, 4th edn, Philadelphia: Lippincott Williams & Wilkins. pp 841–874.

Hale AD, Lewis D, Green J, Jiang X, Brown DWG. 1996. Evaluation of an antigen capture ELISA based on recombinant Mexico virus capsid protein. Clin Diagn Virol 5:27–35.

Hale AD, Crawford SE, Ciarlet M, Green J, Gallimore C, Brown DW, Jiang X, Estes MK. 1999. Expression and self-assembly of Grimsby virus: Antigenic distinction from Norwalk and Mexico viruses. Clin Diagn Lab Immunol 6:142–145.

Jiang X, Wang M, Graham DY, Estes MK. 1992. Expression, self-assembly, and antigenicity of the Norwalk virus capsid protein. J Virol 66:6527–6532.

Jiang X, Wang M, Wang K, Estes MK. 1993. Sequence and genomic organization of Norwalk virus. Virology 195:51–61.

Jiang X, Cubitt D, Hu J, Dai X, Treanor J, Matson DO, Pickering LK. 1995a. Development of an ELISA to detect MX virus, a human calicivirus in the snow mountain agent genogroup. J Gen Virol 76:2739–2747.

Jiang X, Matson DO, Ruiz-Palacios GM, Hu J, Treanor J, Pickering LK. 1995b. Expression, self-assembly, and antigenicity of a snow mountain agent-like calicivirus capsid protein. J Clin Microbiol 33:1452–1455.

Jiang X, Wang J, Estes MK. 1995c. Characterization of SRSVs using RT-PCR and a new antigen ELISA. Arch Virol 140:363–374.

Jiang X, Zhong WM, Farkas T, Huang PW, Wilton N, Barrett E, Fulton D, Morrow R, Matson DO. 2002. Baculovirus expression and antigenic characterization of the capsid proteins of three Norwalk-like viruses. Arch Virol 147:119–130.

Kageyama T, Shinohara M, Uchida K, Fukushi S, Hoshino FB, Kojima S, Takai R, Oka T, Takeda N, Katayama K. 2004. Co-existence of multiple genotypes, including newly identified genotypes, in outbreaks of Norovirus gastroenteritis. J Clin Microbiol 42:2988–2995.

Kapikian AZ. 1994. Norwalk and Norwalk-like viruses. In: Kapikian AZ, editor. Viral infections of the gastroentestinal tract. 2nd edn, New York: Marcel Dekker, Inc. pp 471–518.

Kapikian AZ, Estes MK, Chanock RM. 1996. Norwalk group of viruses. In: Fields BN, Knipe DM, Howley PM, Chanock RM, Melnick JL, Monath TP, Roizmann B, Straus SE, editors. Fields virology. 3rd edn, Philadelphia, PA: Lippincott–Raven Publishers. pp 783–810.

Katayama K, Shirato-Horikoshi H, Kojima S, Kageyama T, Oka T, Hoshino F, Fukushi S, Shinohara M, Uchida K, Suzuki Y, Gojobori T, Takeda N. 2002. Phylogenetic analysis of the complete genome of 18 Norwalk-like viruses. Virology 299:225–239.

Kimura M. 1980. A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. J Mol Evol 16:111–120.

Kitamoto N, Tanaka T, Natori K, Takeda N, Nakata S, Jiang X, Estes MK. 2002. Cross-reactivity among several recombinant calicivirus virus-like particles (VLPs) with monoclonal antibodies obtained from mice immunized orally with one type of VLP. J Clin Microbiol 40:2459–2465.

Kobayashi S, Sakae K, Natori K, Takeda N, Miyamura T, Suzuki Y. 2000a. Serotype-specific antigen ELISA for detection of Chiba virus in stools. J Med Virol 62:233–238.

Kobayashi S, Sakae K, Suzuki Y, Shinozaki K, Okada M, Ishiko H, Kamata K, Suzuki K, Natori K, Miyamura T, Takeda N. 2000b. Molecular cloning, expression, and antigenicity of Seto virus belonging to genogroup I Norwalk-like viruses. J Clin Microbiol 38:3492–3494.

Kobayashi S, Sakae K, Suzuki Y, Ishiko H, Kamata K, Suzuki K, Natori K, Miyamura T, Takeda N. 2000c. Expression of recombinant capsid proteins of chitta virus, a genogroup II Norwalk virus, and development of an ELISA to detect the viral antigen. Microbiol Immunol 44:687–693.

Kojima S, Kageyama T, Fukushi S, Hoshino FB, Shinohara M, Uchida K, Natori K, Takeda N, Katayama K. 2002. Genogroup-specific PCR primers for detection of Norwalk-like viruses. J Virol Methods 100:107–114.

Lambden PR, Caul EO, Ashley CR, Clarke IN. 1993. Sequence and genome organization of a human small round-structured (Norwalk-like) virus. Science 259:516–519.

Lew JF, Kapikian AZ, Jiang X, Estes MK, Green KY. 1994. Molecular characterization and expression of the capsid protein of a Norwalk-like virus recovered from a Desert Shield troop with gastroenteritis. Virology 200:319–325.

Lew JF, Kapikian AZ, Valdesuso J, Green KY. 1994. Molecular characterization of Hawaii virus and other Norwalk-like viruses: Evidence for genetic polymorphism among human caliciviruses. J Infect Dis 170:535–542.

Li TC, Yamakawa Y, Suzuki K, Tatsumi M, Razak MA, Uchida T, Takeda N, Miyamura T. 1997. Expression and self-assembly of empty virus-like particles of hepatitis E virus. J Virol 71:7207–7213.

Numata K, Nakata S, Jiang X, Estes MK, Chiba S. 1994. Epidemiological study of Norwalk virus infections in Japan and Southeast Asia by enzyme-linked immunosorbent assays with Norwalk virus capsid protein produced by the baculovirus expression system. J Clin Microbiol 32:121–126.

Parker S, Cubitt D, Jiang JX, Estes M. 1993. Efficacy of a recombinant Norwalk virus protein enzyme immunoassay for the diagnosis of infections with Norwalk virus and other human "candidate" caliciviruses. J Med Virol 41:179–184.

Parker SP, Cubitt WD, Jiang XJ, Estes MK. 1994. Seroprevalence studies using a recombinant Norwalk virus protein enzyme immunoassay. J Med Virol 42:146–150.

Parker SP, Cubitt WD, Jiang X. 1995. Enzyme immunoassay using baculovirus-expressed human calicivirus (Mexico) for the measurement of IgG responses and determining its seroprevalence in London, UK. J Med Virol 46:194–200.

Prasad BV, Rothnagel R, Jiang X, Estes MK. 1994. Three-dimensional structure of baculovirus-expressed Norwalk virus capsids. J Virol 68:5117–5125.

Prasad BV, Hardy ME, Dokland T, Bella J, Rossmann MG, Estes MK. 1999. X-ray crystallographic structure of the Norwalk virus capsid. Science 286:287–290.

Saitou N, Nei M. 1987. The neighbor-joining method: A new method for reconstructing phylogenetic trees. Mol Biol Evol 4:406–425.

Vipond IB, Caul EO, Hirst D, Carmen B, Curry A, Lopman BA, Pead P, Pickett MA, Lambden PR, Clarke IN. 2004. National epidemic of Lordsdale Norovirus in the UK. J Clin Virol 30:243–247.

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Genetic and antigenic diversity among noroviruses

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Human norovirus (NoV) strains cause a considerable number of outbreaks of gastroenteritis worldwide. Based on their capsid gene (VP1) sequence, human NoV strains can be grouped into two genogroups (GI and GII) and at least 14 GI and 17 GII genotypes (GI/1–14 and GII/1–17). Human NoV strains cannot be propagated in cell-culture systems, but expression of recombinant VP1 in insect cells results in the formation of virus-like particles (VLPs). In order to understand NoV antigenic relationships better, cross-reactivity among 26 different NoV VLPs was analysed. Phylogenetic analyses grouped these NoV strains into six GI and 12 GII genotypes. An antibody ELISA using polyclonal antisera raised against these VLPs was used to determine cross-reactivity. Antisera reacted strongly with homologous VLPs; however, a number of novel cross-reactivities among different genotypes was observed. For example, GI/11 antiserum showed a broad-range cross-reactivity, detecting two GI and 10 GII genotypes. Likewise, GII/1, GII/10 and GII/12 antisera showed a broad-range cross-reactivity, detecting several other distinct GII genotypes. Alignment of VP1 amino acid sequences suggested that these broad-range cross-reactivities were due to conserved amino acid residues located within the shell and/or P1-1 domains. However, unusual cross-reactivities among different GII/3 antisera were found, with the results indicating that both conserved amino acid residues and VP1 secondary structures influence antigenicity.

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INTRODUCTION

Norovirus (NoV) strains are a leading cause of gastroenteritis worldwide and cause outbreaks in various epidemiological settings including hospitals, cruise ships, schools and restaurants (Beuret *et al.*, 2003; Inouye *et al.*,

The GenBank/EMBL/DDBJ accession numbers of the sequences reported in this paper are AB081723, AB058547, DQ093065, AB195225, DQ093062, DQ093066, DQ093063, AB195226, DQ093064, AB039780, AY237414 and DQ093067.

2000; Johansson *et al.*, 2002; Kapikian *et al.*, 1972; McEvoy *et al.*, 1996; McIntyre *et al.*, 2002; Russo *et al.*, 1997). Numerous molecular epidemiological studies have revealed a global distribution of these viruses (Nakata *et al.*, 1998; Noel *et al.*, 1999; White *et al.*, 2002). Transmission routes include food-borne, person-to-person contact and environmental contamination. Human NoV can be divided into two genetically distinct genogroups, GI and GII. Recently, NoV strains were subdivided into at least 14 GI and 17 GII genotypes (GI/1–14 and GII/1–17) (Kageyama *et al.*, 2004). These viruses cannot be grown in culture and their antigenic relationships are not completely understood. Nevertheless, expression of the major capsid protein (VP1), which usually results in the formation of virus-like particles (VLPs) that are morphologically similar to the native virus, has permitted a better understanding of antigenicity in these viruses (Hansman *et al.*, 2004). Two types of assay have been used to examine cross-reactivity among these VLPs: antibody ELISA and antigen ELISA (Gray *et al.*, 1993; Jiang *et al.*, 1995a, b; Kageyama *et al.*, 2004; Kamata *et al.*, 2005; Kobayashi *et al.*, 2000a, b, c). The antibody ELISA is broadly reactive, but the antigen ELISA is highly specific, only detecting strains that are closely related (> 95 % identity in the RNA polymerase region). However, detailed information on the cross-reactivity among many of the genetically distinct NoV strains is limited.

NoVs are small round viruses approximately 38 nm in diameter and possess a single-stranded, positive-sense RNA genome of 7·5–7·7 kb. The NoV genome contains three open reading frames (ORFs). ORF1 encodes non-structural proteins, including the RNA-dependent RNA polymerase, ORF2 encodes VP1 and ORF3 encodes a minor capsid protein (VP2) (Jiang *et al.*, 1990). Cryo-electron microscopy (cryo-EM) and X-ray crystallography analyses of NoV VLPs have determined the shell and protruding domains (subdomains P1-1, P1-2 and P2) of the capsid protein (Prasad *et al.*, 1999). Chen *et al.* (2004) also described strictly and moderately conserved amino acid residues in the capsid protein among the four genera in the family *Caliciviridae*.

The aim of this study was to analyse cross-reactivity among 26 different NoV VLPs in order to understand NoV genetic and antigenic relationships in more detail. An antibody ELISA using polyclonal antisera raised against the VLPs was used to determine cross-reactivities. Our results found broad-range cross-reactivities with antisera raised against a number of distinct NoV strains.

METHODS

Specimens. Positive stool specimens were collected from a number of different sources (see GenBank accession numbers) and RNA was extracted as described previously (Katayama *et al.*, 2002). PCR-generated amplicons or plasmids were excised from the gel and purified by using the QIAquick Gel Extraction kit and Plasmid Purification kit (Qiagen). Nucleotide sequences were prepared with the BigDye Terminator Cycle Sequence kit (version 3.1) (Applied Biosystems) and determined by using the ABI 3100 Avant sequencer (Perkin-Elmer ABI). Nucleotide sequences were aligned with CLUSTAL_X and distances were calculated using Kimura's two-parameter method. Phylogenetic trees with bootstrap analysis from 1000 replicas were generated by the neighbour-joining method as described previously (Kageyama *et al.*, 2004). Amino acid VP1 secondary structure predictions were made using the PSIPRED secondary structure prediction software (McGuffin *et al.*, 2000).

Expression of VLPs. Previously, we expressed four GI NoV strains: GI/1 (strain SeV), GI/2 (strain 258), GI/3 (strain 645) and GI/4 (strain CV), and nine GII NoV strains: GII/3 (strain 809), GII/4 (strain 104), GII/5 (strain 754), GII/6 (strain 7k), GII/7 (strain 10-25), GII/10 (strain 026), GII/12 (strains CHV and 9912-02F; in this study 9912-02F was termed Hiro) and GII/14 (strain 47) (Hansman *et al.*, 2004; Kamata *et al.*, 2005; Kitamoto *et al.*, 2002; Kobayashi *et al.*, 2000a, b, c). Dr Kim Green provided us with the Hawaii virus recombinant baculovirus GII/1 (strain HV) (Green *et al.*, 1997). In this study, we expressed an additional 12 VLPs: GI/8 strain WUG1 (using primers G1SKF and TX30SXN; see Table 1 for primer sequences); GI/11 strain

Table 1. Primer sequences used for expression of VLPs

Primer	Sense/antisense	Sequence (5′–3′)*
G1SKF	Sense	CTGCCCGAATTYGTAAATGA
TX30SXN	Antisense	GACTAGTTCTAGATCGCGAGCGGCCGCCC$(T)_{30}$
G2/F3	Sense	TTGTGAATGAAGATGGCGTCGA
G2R0	Antisense	CCATTACTGAACCCTTCTACGCC
G2Fb	Sense	TGGGAGGGCGATCGCAATCT
G2R04	Antisense	GGCGTAGAAGGYTTCAYTAAGTC
MVR1	Antisense	AATTATTGAATCCTTCTACGCCCG
G2F2	Sense	GTGAATGAAGATGGCGTCGA
G2R03	Antisense	GGCGTAGAAGGATTCAATAATGG
G2F02	Sense	GTGAATGAAGATGGCGTCGAATGA
G2SKF	Sense	CNTGGGAGGGCGATCGCAA
NAL13	Sense	GATCTCGCTCCCGATTTTTGTGA
N235R	Antisense	ATGGCWGGAGCTTTRATAGC

*Y=C or T; N=A, C, G or T; W=A or T.

#8 (primers G1SKF and TX30SXN); GII/1 strain 485 (primers G2/F3 and G2R0); GII/2 strain Ina (primers G2Fb and G2R04); GII/3 strain 18-3 (primers G2/F3 and MVR1); GII/3 strain 1152 (primers G2F2 and G2R03); GII/3 strain 336 [primers G2/F3 and Oligo-$(dT)_{33}$]; GII/3 strain Sh5 (primers G2F02 and G2R03); GII/6 strain 445 [primers G2/F3 and Oligo-$(dT)_{33}$]; GII/8 strains Mc24 and U25 (primers G2SKF and TX30SXN); and GII/17 strain Alph23 (primers NAL13 and N235R). For expression of the recombinant VP1 in insect cells, all of the NoV constructs were designed to begin from the predicted VP1 AUG start codon. For six of the 12 constructs, the VP2 and poly(A) sequences were included by using either the TX30SXN or Oligo-$(dT)_{33}$ reverse primers (strains WUG1, #8, 336, 445 and Mc24). One construct (strain 485) excluded the poly(A) sequence, whereas the remaining five constructs excluded both the VP2 and poly(A) sequences (strains Alph23, Sh5, 1152 and 18-3 and Ina). Four constructs that were amplified with the TX30SXN reverse primer were expressed using the Gateway expression system (strains WUG1, #8, U25 and Mc24) (Hansman *et al.*, 2004), whilst the other eight constructs were expressed in a baculovirus expression system as described previously (Kamata *et al.*, 2005).

VLP purification and electron microscopy (EM). Recombinant baculovirus shuttle vectors (bacmids) were transfected into Sf9 cells using Effectene according to the manufacturer's instructions (Qiagen). Sf9 cells were incubated for 5–6 days at 26 °C, after which the culture medium was clarified by low-speed centrifugation and the supernatant was stored as the seed baculovirus. Tn5 cells were infected with the seed baculovirus at 26 °C and harvested 5–6 days post-infection. VLPs secreted into the cell medium were separated from cells by low-speed centrifugation, concentrated by ultracentrifugation at 30 000 r.p.m. at 4 °C for 2 h (Beckman SW-32 rotor) and then resuspended in 100 µl Grace's medium. VLPs were purified by CsCl equilibrium gradient ultracentrifugation at 45 000 r.p.m. at 15 °C for 18 h (Beckman SW-55 rotor). The harvested culture medium was examined for VLPs by negative-staining EM. Briefly, the samples (diluted 1 : 10 in distilled water) were applied to a carbon-coated 300-mesh EM grid and stained with 2 % uranyl acetate (pH 4). Grids were examined under an electron microscope (JEM-1220; JEOL) operating at 80 kV.

Antibody production and ELISA. Hyperimmune sera to newly developed VLPs were prepared in rabbits. The first subcutaneous injection was performed with purified VLPs (between 10 and 500 µg) in Freund's complete adjuvant. After 3 weeks, the animals received two or three booster injections of the same amount of VLPs in Freund's incomplete adjuvant at intervals of 1 week. The animals were bled 1 week after the last booster injection. An antibody ELISA was used to compare cross-reactivities among the VLPs. Then wells of 96-well microtitre plates (Maxisorp; Nunc) were each coated with 100 µl purified VLPs (1·0 µg ml^{-1} in carbonate/bicarbonate buffer, pH 9·6; Sigma) and incubated overnight at 4 °C. The wells were washed twice with PBS containing 0·1 % (v/v) Tween 20 (PBS-T)

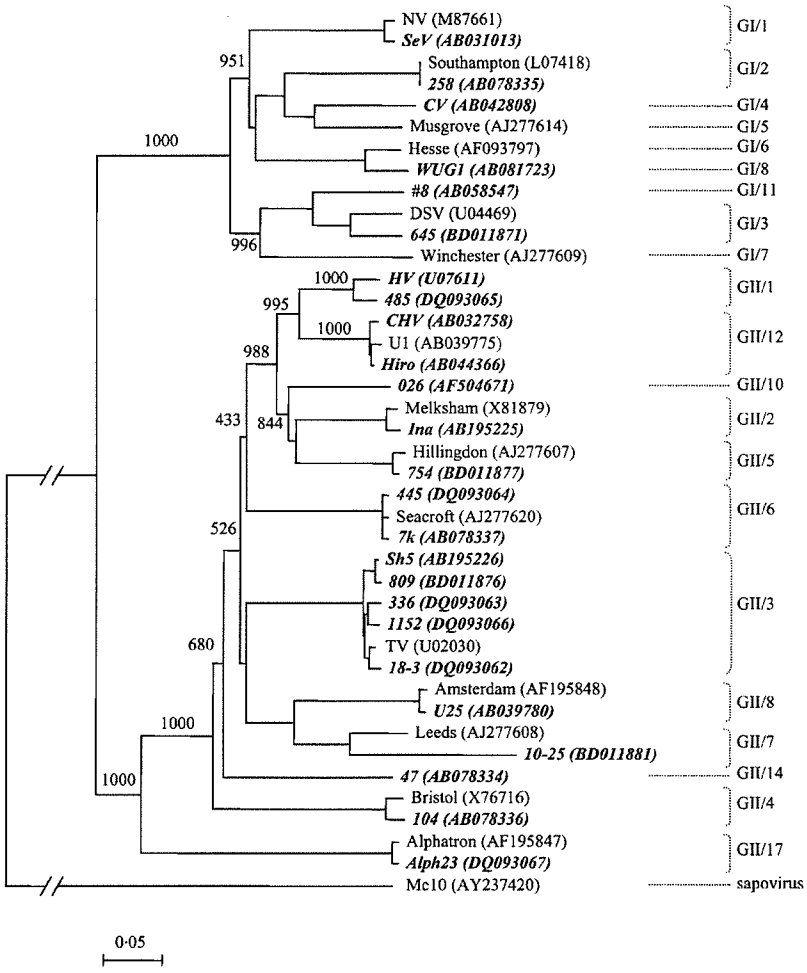


Fig. 1. Phylogenetic tree of NoV sequences examined in this study (shown in bold italic). NoV amino acid sequences were constructed using the entire VP1 sequence (the complete sequence for Mc24 was unavailable). Numbers on branches indicate bootstrap values for the clusters; values of 950 or higher were considered statistically significant for the grouping (Katayama *et al.*, 2002). Reference sequences have been reported previously (Kageyama *et al.*, 2004).

and then blocked with PBS containing 5% (w/v) skimmed milk (PBS-SM) for 1 h at room temperature. After the wells had been washed twice with PBS-T, 100 µl twofold serially diluted hyperimmune rabbit antiserum from a starting dilution of 1:2000 in PBS-T-SM was added to each well and the plates were incubated for 1 h at 37 °C. The wells were washed six times with PBS-T and 100 µl horseradish peroxidase-conjugated anti-rabbit IgG (1:1000 dilution in PBS-T-SM) was added to each well. The plates were incubated for 1 h at 37 °C. The wells were washed six times with PBS-T and 100 µl *o*-phenylenediamine substrate and H_2O_2 was added to each well. The plates were left in the dark for 30 min at room temperature. The reaction was stopped by the addition of 50 µl 1 M H_2SO_4 to each well and the absorbance was measured at 492 nm. ELISA titres were expressed as the reciprocal of the highest dilution of antiserum giving a value of $A_{492} > 0{\cdot}2$.

RESULTS

Sequence analysis

Nucleotide and amino acid sequences were aligned using CLUSTAL_X and distances were calculated using Kimura's two-parameter method. We divided the 26 strains used in this study into six GI and 12 GII genotypes using partial N-terminal VP1 nucleotide sequences (data not shown). These genotypes were maintained when we grouped the complete VP1 amino acid sequences (Fig. 1). Mc24 was excluded from the amino acid analysis since the full-length capsid sequence was unavailable. Nevertheless, using the partial N-terminal VP1 nucleotide sequence (GenBank accession no. AY237414), Mc24 clustered in GII/8 and was closely related to strain U25. Of the recently described NoV strains (Kageyama *et al.*, 2004), the GI and GII genotypes used in this study represented 43% (6/14) and 76% (13/17), respectively. For several GII genotypes, we used two or more VLPs in order to clarify antigenicity, including GII/1 (strains HV and 485), GII/3 (strains 809, Sh5, 336, 1152 and 18-3), GII/6 (strains 7k and 445) and GII/12 (strains CHV and Hiro).

Expression of VP1

EM confirmed that all strains formed VLPs with morphological features similar to native NoV (Fig. 2), despite the fact that different constructs and expression systems were used to express the recombinant VP1. The VLPs retained their morphological features, even when stored for >6 months at −20 °C (data not shown).

Homologous antigenic analysis

An antibody ELISA, which uses polyclonal antiserum raised against one type of VLP, was used to determine cross-reactivity among the 26 different NoV VLPs. ELISA titres were expressed as the reciprocal of the highest dilution of antiserum giving a value of $A_{492} > 0{\cdot}2$. A negative control (baculovirus-infected Tn5 cell lysate) was used for all experiments and found to be negligible [i.e. $A_{492} < 0{\cdot}05$, using up to 0·5 µg (ml lysate)$^{-1}$]. Antisera reacted strongly against homologous VLPs, with titres ranging from 102 400 to 1 638 400 (Table 2).

Heterologous antigenic analysis

We observed a number of novel cross-reactivities among different genotypes. For example, Fig. 3(a) shows the strong cross-reactivity of GI/11 #8 antiserum with both GII/6 7k and GII/6 445 VLPs. We found that GI/11 #8 antiserum cross-reacted with these GII/6 VLPs at titres of 102 400,

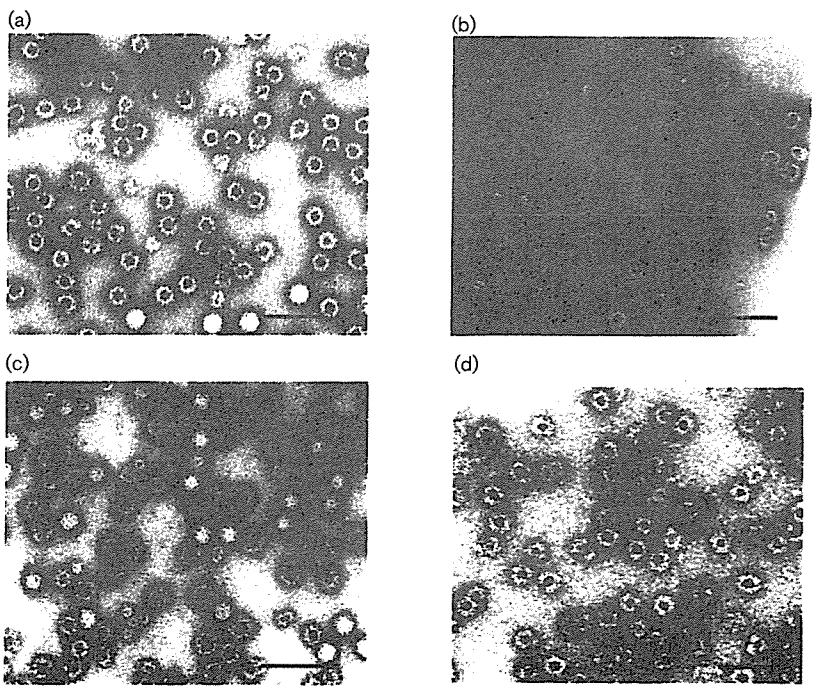
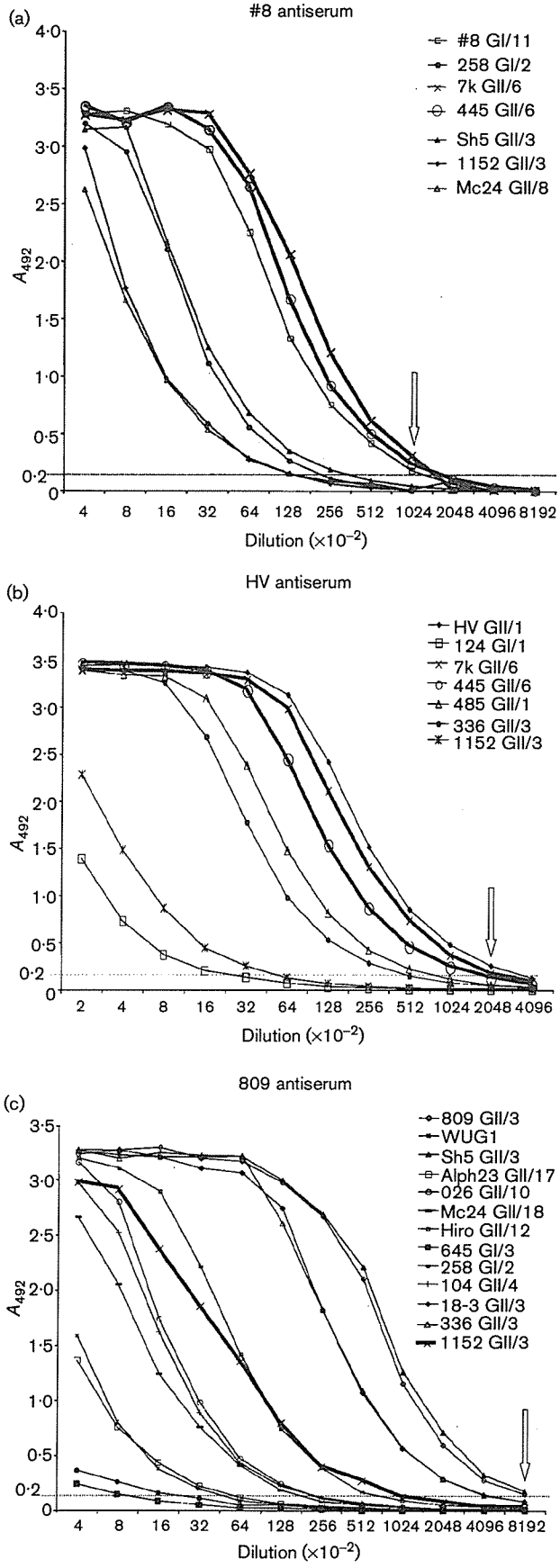


Fig. 2. EM images of CsCl-purified NoV VLPs negatively stained with 2% uranyl acetate (pH 4). (a) Strain 7k, (b) strain 485, (c) strain 445 and (d) strain 645. Bar, 100 nm.

Table 2. Antibody ELISA cross-reactivity among the 26 different VLPs and antisera

Titres are expressed as $\times 10^{-2}$.

Genogroup			GI						GII																			
	Genotype	VLPs	1	2	3	4	8	11	1	1	2	3	3	3	3	3	4	5	6	6	7	8	8	10	12	12	14	17
	Antiserum	Strains	SeV	258	645	CV	WUG1	#8	HV	485	Ina	809	Sh5	18-3	336	1152	104	754	7k	445	10-25	Mc24	U25	026	CHV	Hiro	47	Alph23
GI	1	SeV	**8192**	1042	1024	1024	1024	1024	256	128	256	256	256	256	256	128	128	256	256	256	128	128	256	256	256	256	256	256
	2	258	512	**8192**	512	1024	512	512	64	16	64	32	128	64	32	16	128	128	64	64	32	32	64	128	32	64	32	128
	3	645	256	256	**8192**	256	512	1024	32	8	32	64	128	128	64	64	64	64	32	64	128	128	128	128	16	32	16	64
	4	CV	128	512	256	**8192**	512	128	64	4	32	64	128	64	32	64	32	32	32	32	64	64	64	64	16	64	32	64
	8	WUG1	256	512	256	256	**8192**	256	32	32	16	32	64	64	64	16	32	32	32	32	64	8	32	32	32	16	32	64
	11	#8	128	128	128	256	256	**1024**	256	128	256	256	256	256	256	128	256	256	1024	1024	256	128	128	256	128	256	128	256
GII	1	HV	16	16	8	16	32	256	**2048**	512	128	128	256	512	512	32	256	256	2048	1024	128	128	128	256	512	512	512	256	256
	1	485	128	128	16	128	128	128	1024	**4096**	128	256	256	256	256	64	128	256	128	128	128	64	256	256	512	256	256	128
	2	Ina	<4	32	16	16	64	32	256	256	**4096**	128	128	256	128	64	512	512	256	128	128	256	128	512	256	512	128	128
	3	809	<2	4	4	8	32	4	512	128	128	**8192**	8192	4096	4096	1024	64	256	64	128	256	128	256	128	256	512	64	32
	3	Sh5	8	16	16	16	16	16	128	64	64	2048	**2048**	1024	1024	256	128	128	64	64	128	64	128	128	128	128	64	64
	3	18-3	16	16	16	16	32	32	64	64	64	256	512	**1024**	512	128	64	64	64	64	128	64	128	128	128	128	64	64
	3	336	32	16	32	32	32	64	128	128	128	1024	1024	1024	**2048**	512	128	128	128	128	256	128	256	128	256	256	128	128
	3	1152	<4	<4	<4	<4	8	<4	16	16	32	512	1024	1024	1024	**1024**	32	64	32	32	32	32	32	32	16	32	8	16
	4	104	32	64	128	64	256	128	512	512	512	512	512	1024	1024	128	**8192**	1024	256	512	512	256	512	512	512	512	512	256
	5	754	128	128	64	128	256	512	1024	1024	1024	512	1024	1024	1024	256	512	**8192**	512	1024	512	256	512	1024	1024	1024	512	512
	6	7k	64	128	64	128	128	1024	512	128	128	128	256	256	128	32	256	256	**4096**	2048	128	64	256	256	256	256	128	256
	6	445	16	16	8	16	32	128	64	32	64	128	128	128	128	64	64	64	2048	**4096**	64	64	64	128	64	64	64	128
	7	10-25	32	64	128	128	64	128	512	512	256	1024	512	1024	512	128	512	512	512	512	**16384**	512	1024	512	512	512	512	256
	8	Mc24	8	16	4	8	16	NT	64	32	NT	16	NT	32	32	8	32	32	16	16	128	**2048**	2048	32	32	32	16	4
	8	U25	16	32	32	32	32	32	256	256	128	128	256	256	256	64	256	256	128	128	512	4096	**4096**	256	256	256	128	128
	10	026	32	32	32	64	128	16	512	512	256	128	128	256	256	128	256	512	256	256	512	256	256	**2048**	512	512	256	128
	12	CHV	32	64	64	64	128	128	1024	1024	512	256	256	512	64	512	256	512	256	256	256	128	512	1024	**4096**	2048	512	256
	12	Hiro	<2	16	4	2	8	4	256	128	64	64	64	64	64	16	64	128	32	64	64	32	64	128	1024	**1024**	64	32
	14	47	128	128	64	256	256	256	512	256	512	512	512	512	512	128	512	512	256	256	256	128	512	512	512	512	**4096**	256
	17	Alph23	64	64	32	32	8	32	64	32	128	32	128	64	32	256	64	64	64	64	256	64	128	256	64	256	32	**2048**



which was equal to the homologous VLP titre. We also found that GII/1 HV antiserum cross-reacted strongly (i.e. equal to the homologous VLP titre) against GII/6 7k VLPs (titre 204 800) and moderately strongly (i.e. twofold lower than the homologous VLP titre) against GII/6 445 VLPs (titre 102 400) (Fig. 3b and Table 2). We observed several antisera that cross-reacted moderately against different genotypes (i.e. fourfold lower than the homologous VLP titres). For example, GI/11 #8 antiserum cross-reacted moderately with GI/4, GI/8, GII/1, GII/2, GII/3, GII/4, GII/5, GII/7, GII/10, GII/12 and GII/17 VLPs (Fig. 3a and Table 2). GII/1 HV antiserum also cross-reacted moderately with several different genotypes, including GII/1 (strain 485), GII/3, GII/10 and GII/12 (Fig. 3b and Table 2). GII/1 485 antiserum cross-reacted moderately only with GII/1 HV VLPs; GII/6 7k antiserum cross-reacted moderately with GI/11 VLPs; GII/10 026 antiserum cross-reacted moderately with several different genotypes, including GII/1, GII/5, GII/7 and GII/12; and GII/12 CHV antiserum cross-reacted moderately with GII/1 and GII/10 VLPs (Table 2).

Genotype-specific reactivities

We observed weak cross-reactivities among different genotypes (i.e. greater than eightfold dilutions). We found that GI/1, GI/2, GI/3, GI/4 and GI/8 antisera cross-reacted weakly with other genotypes (Table 2). We also observed similar weak cross-reactivities with GII/1 (strain 485), GII/2, GII/3 (all five strains), GII/4, GII/5, GII/6 (strain 445), GII/7, GII/8 (both strains), GII/14 and GII/17 antisera. For several GII genotypes, only one type of antiserum was produced, but for five GII genotypes, we produced two or more different antisera against VLPs belonging to the same genotype (Table 2). Some interesting results were observed. For example, the antigenicities of HV and 485 were considerably different, despite the fact that both strains belong to GII/1 and share approximately 94% amino acid identity. As shown in Fig. 3(b), HV antiserum cross-reacted strongly with GII/6 VLPs, but 485 antiserum showed little cross-reactivity with these GII/6 VLPs (Table 2). This unusual cross-reactivity pattern was also observed with other antisera. For example, for GII/6, we found that 7k antiserum cross-reacted moderately with GI/11 #8 VLPs, whereas 445 antiserum cross-reacted weakly (i.e. 32-fold lower than the homologous VLP titre; Table 2). More uniquely, we found that GII/3 1152 antiserum, which was genotype-specific, had unusual antigenicity. We found that

Fig. 3. Antibody ELISAs for NoV VLPs. Wells were coated with 100 µl purified VLPs. After washing, hyperimmune rabbit antiserum raised against the VLPs was used to detect antigens. Antisera were diluted twofold in PBS-T-SM from a starting dilution as indicated (dilutions × 10^{-2}). The arrows indicate the endpoint. (a) GI/11 #8 antiserum cross-reacts strongly with GII/6 7k and 445 VLPs. (b) GII/1 HV antiserum cross-reacts strongly with GII/6 7k and moderately strongly with 445 VLPs. (c) GII/3 809 antiserum cross-reacts weakly with GII/3 1152 VLPs.

three different GII/3 antisera (strains 809, Sh5 and 18-3) cross-reacted weakly with 1152 VLPs (i.e. eightfold lower than the homologous VLP titre; Table 2 and Fig. 3c). This unusual cross-reactivity result was not evident with the other genotypes in which we produced two different antisera (i.e. GII/1, GII/6, GII/8 and GII/12; see Table 2).

Amino acid alignment and secondary structure prediction

An alignment of 25 VP1 amino acid sequences used in this study (Mc24 complete capsid was unavailable) revealed that the N-terminal region (aa 1–49), shell domain (aa 50–225) and P1-1 domain (aa 226–278) had more conserved short continuous residues than the P2 domain (aa 279–405), P1-2 domain (aa 406–520) and C-terminal region (Fig. 4). These continuous residues may be the reason for the cross-reactivity among different genotypes, in particular, the strong cross-reactivity of #8 antiserum against GII/6 VLPs (Fig. 3a). However, this does not explain why GII/3 1152 VLPs cross-reacted weakly with GII/3 809, Sh5 and 18-3 antisera (i.e. eightfold lower than the homologous VLP titre) and moderately against GII/3 336 antiserum (i.e. fourfold lower than the homologous VLP titre). An amino acid

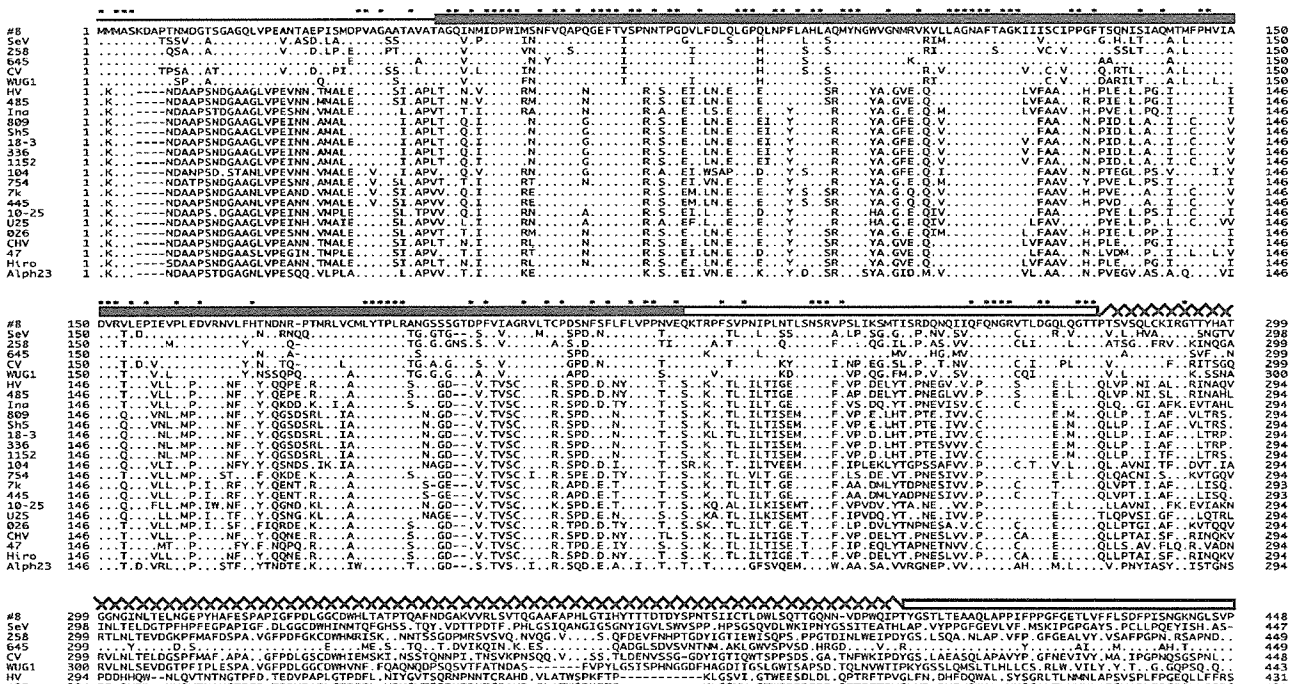
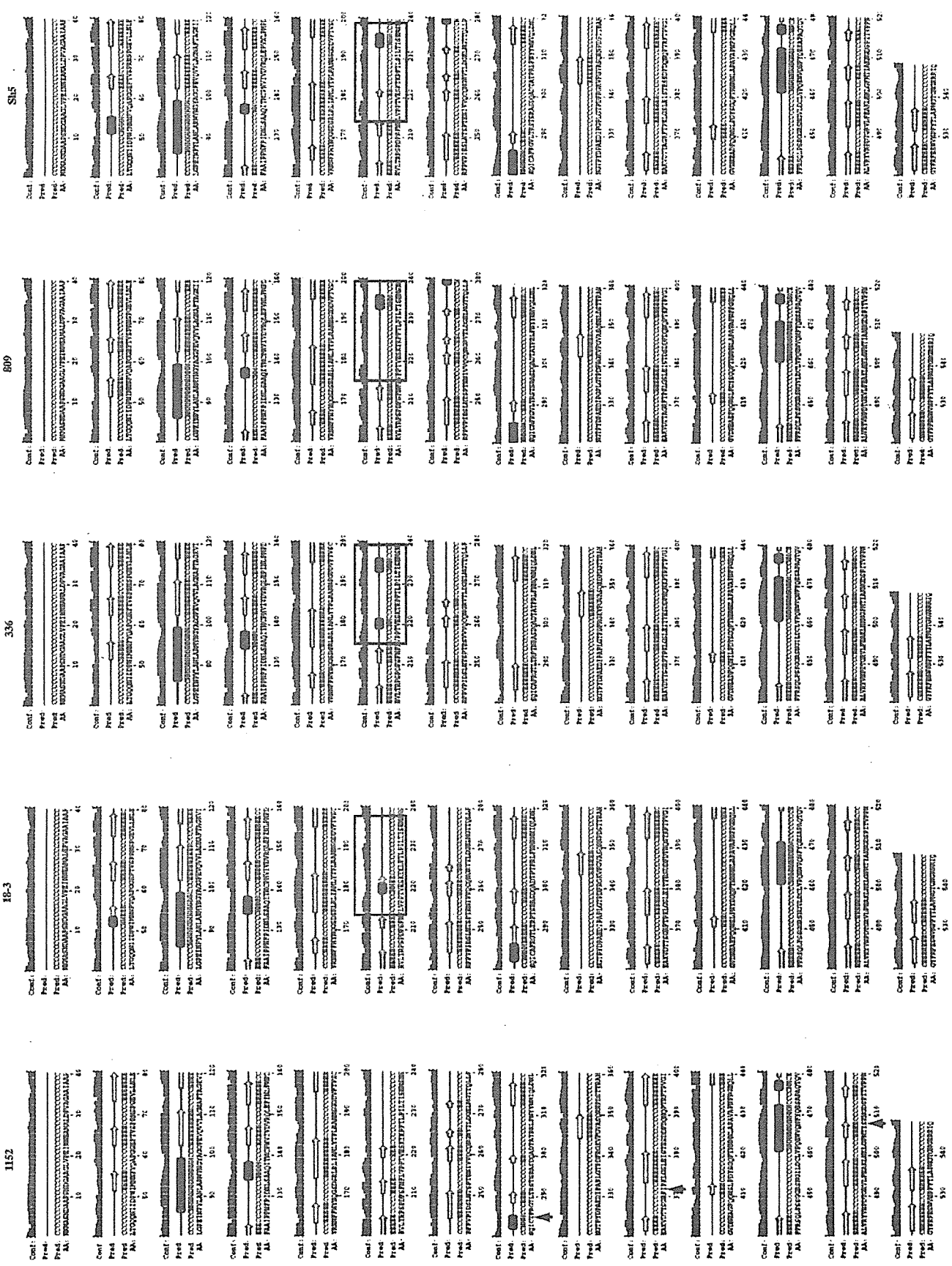


Fig. 4. Amino acid alignment of VP1 sequences of the NoV sequences examined in this study. The following regions are indicated above the sequences (in order): N-terminal region (line); shell domain (filled box), P1-1 domain (open box); P2 domain (XXX); P1-2 domain (open box) and C-terminal region (line) (Chen *et al.*, 2004). Asterisks indicate conserved amino acids.


1152
18-3
336
809
Sb5