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Isolation of Human Monoclonal Antibodies That Neutralize Human Rotavirus

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A human antibody library constructed by utilizing a phage display system was used for the isolation of human antibodies with neutralizing activity specific for human rotavirus. In the library, the Fab form of an antibody fused to truncated cp3 is expressed on the phage surface. Purified virions of strain KU (G1 serotype and P[8] genotype) were used as antigen. Twelve different clones were isolated. Based on their amino acid sequences, they were classified into three groups. Three representative clones—1-2H, 2-3E, and 2-11G—were characterized. Enzyme-linked immunosorbent assay with virus-like particles (VLP-VP2/6 and VLP-VP2/6/7) and recombinant VP4 protein produced from baculovirus recombinants indicated that 1-2H and 2-3E bind to VP4 and that 2-11G binds to VP7. The neutralization epitope recognized by each of the three human antibodies might be human specific, since all of the antigenic mutants resistant to mouse monoclonal neutralizing antibodies previously prepared were neutralized by the human antibodies obtained here. After conversion from the Fab form of an antibody into immunoglobulin G1, the neutralizing activities of these three clones toward various human rotavirus strains were examined. The 1-2H antibody exhibited neutralizing activity toward human rotaviruses with either the P[4] or P[8] genotype. Similarly, the 2-3E antibody showed cross-reactivity against HRVs with the P[6], as well as the P[8] genotype. In contrast, the 2-11G antibody neutralized only human rotaviruses with the G1 serotype. The concentration of antibodies required for 50% neutralization ranged from 0.8 to 20 μg/ml.

Rotavirus is the major cause of severe acute gastroenteritis among infants and young children. Rotavirus infection is life-threatening in developing countries, resulting in 500,000 to 600,000 deaths annually (33). In developed countries, rotavirus infections lead to a high disease burden with considerable medical expense due to the high morbidity. Furthermore, adults, particularly the elderly, are also affected by rotavirus infection (34, 39), and immunocompromised children and adults develop persistent rotavirus diarrhea (12, 42, 43). Thus, vaccination is thought to be the best way to reduce severe rotavirus gastroenteritis worldwide. Tetravalent rhesus rotavirus (RRV) human reassortant vaccine comprising RRV and three RRV-based monoreassortants carrying the VP7 genes from G1, G2, and G4 human rotaviruses (HRVs) was developed (25), and 1.5 million doses of this vaccine had been administered to infants by the end of May 1999 in the United States. However, the vaccine was withdrawn due to the occurrence of gut intussusception, which appeared to be epidemiologically linked to vaccine application (5, 38). Moreover, even if a safe and effective rotavirus vaccine is developed, vaccination would be less effective in immunocompromised patients.

Rotaviruses have two outer capsid proteins, viral protein 4 (VP4) and VP7, encoded on RNA segment 4 and RNA segment 7, 8, or 9, depending on the strain, respectively (19). VP4 and VP7 are known to induce neutralizing antibodies (Abs) in the sera and stools of infected patients, and they are relevant to protection against rotavirus infection (14, 18, 41, 45–47, 51). It is well known that the rotavirus G serotypes and P genotypes defined by VP7 and VP4, respectively, exhibit diversity. A total of 15 G serotypes and 22 P genotypes have been described (11). Although the majority of HRVs prevailing worldwide have G1, G2, G3, or G4 as the G serotype, and P[4] or P[8] as the P genotype, at least 10 G and 10 P types have been reported on HRVs (8). Recently, a number of HRV strains with unusual G or P types and rare combinations of G and P types have been detected worldwide. For example, G9 is increasing rapidly. In contrast, both VP4 and VP7 carry heterotypic (cross-reactive) neutralization epitopes, which are thought to be related to heterotypic protection (29, 30, 45–47, 49). An individual can be repeatedly infected with various strains of HRVs, suggesting that he or she has broadly and strongly effective Abs to HRVs. Although the validity of passive immunization remains unclear (17), oral administration of cross-reactive human immunoglobulins could be one of the measures for both prophylaxis and therapy for HRV diseases.

The natural repertoire formed in the human body should be composed of two different types of Abs. One type, which forms a naive repertoire, should show a wide range of antigen (Ag) specificity, the Ab binding avidity of each Ab being low in general. The other type, which is raised against specific Ags by immunization, should show a narrow range of specificity, the Ab binding avidity of each Ab being strong. In the present study, we used the Ab library called AIMS4 constructed from the B lymphocyte-rich tissues of a few dozen patients. Since this library is human-derived and rotavirus infection is considered to be very common worldwide, we expected that the Ab

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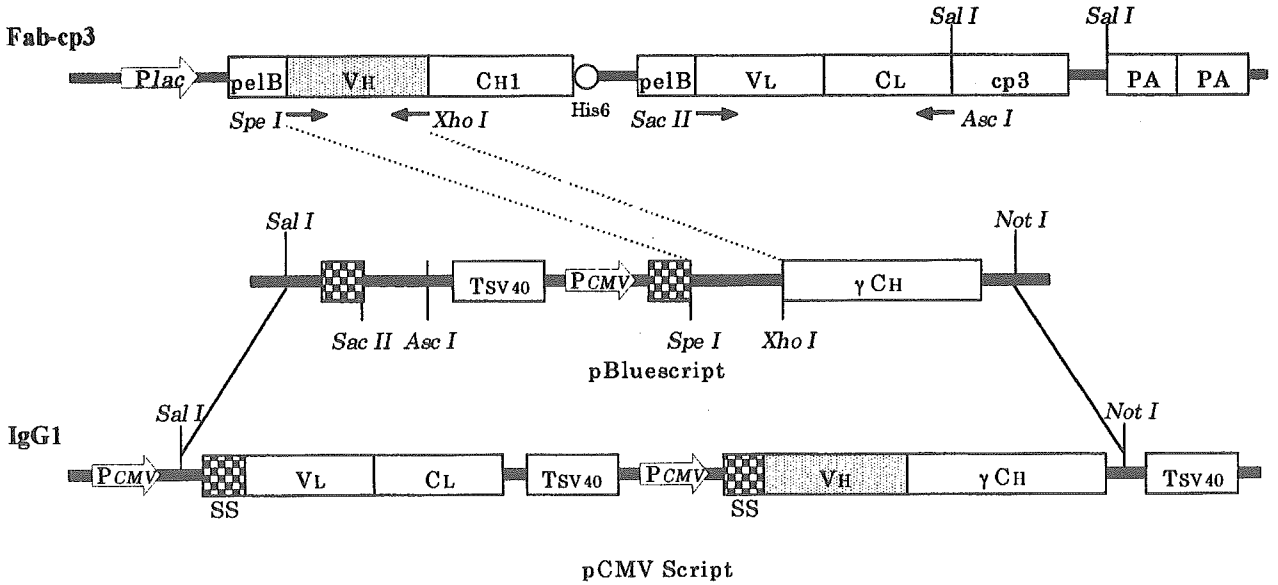

FIG. 1. Scheme for the conversion of a phage Ab (Fab-cp3) to an human IgG1. The V_H and the V_L C_L regions were amplified by PCR with the proper primers listed in Table 1, followed by subcloning into the proper restriction enzyme sites to construct an IgG1 cassette. The IgG1 cassette was then cloned into expression vector pCMVScript. *Plac*, *lac* promoter; *pel*B, *pel*B leader sequence; C_H1, the first H-chain constant domain of human IgG1; His6, His tag-encoding part; cp3, truncated cp3; PA, Fc-binding domain of protein A; Tsv40, simian virus 40 terminator; P_{CMV}, cytomegalovirus promoter; γC_H1, human γC_H1 domain; SS, signal sequence.

repertoire formed in AIMS4 should reflect a variety of rotavirus-specific Abs acquired through natural exposure. It would be interesting to directly explore the Ab repertoire of humans. In particular, comparison of the neutralization epitopes recognized by humans and mice would be useful for understanding the immune response against rotavirus infection in humans.

We describe here the successful isolation of anti-VP4 cross-reactive Abs and an anti-VP7 G1-specific Ab with neutralizing activities toward rotaviruses.

MATERIALS AND METHODS

Viruses. The following HRV strains and reassortants were used for the present study: KU (G1P[8]), Wa (G1P[8]), M37 (G1P[6]), K8 (G1P[9]), S2 (G2P[4]), 1076 (G2P[6]), YO (G3P[8]), McN13 (G3P[6]), AU-1 (G3P[9]), Hosokawa (G4P[8]), 69M (G8P[10]), WI61 (G9P[8]), L26 (G12P[4]), and two bovine strain UK-based single gene-reassortants, UK/Wa (G1P[5]) and UK/DS1 (G2P[5]), carrying the VP7 gene from HRV strains Wa and DS1, respectively (32). Eleven antigenic KU mutants resistant to each of 11 neutralizing mouse monoclonal Abs (MAbs) were also used in the present study: six (V-YO-1E6, V-ST-1F2, V-YO-1S3, V-YO-2C2, V-KU-4D7, and V-KU-6B11) and five (V-KU-3C7, V-YO-4C2, V-KU-5H1, V-KU-6A11, and V-KU4) mutants have been prepared by cultivating strain KU in the presence of anti-VP4 cross-reactive neutralizing MAbs and anti-VP7 G1-specific neutralizing MAbs, respectively (45, 47). Virus propagation and purification were carried out as described previously (49). Unless otherwise stated, the culture fluids of MA104 cells infected with rotaviruses were used for the assays.

Preparation of virus-like particles (VLPs) and recombinant VP4. Construction of the artificial VLP of HRV KU origin is described elsewhere (K. Taniguchi et al., unpublished data). Briefly, the reverse transcription-PCR products of the VP2, VP4, VP6, and VP7 genes of human strain KU were cloned into a TA cloning vector, pCRII (Invitrogen, San Diego, Calif.), to generate pKU-VP2, pKU-VP4, pKU-VP6, and pKU-VP7. After digestion with restriction enzymes, the fragments were ligated into transfer vector pVL1392 to yield pVL1392/KU-VP2, -VP4, -VP6, and -VP7. Sf9 cells were coinfected with linearized wild-type *Autographa californica* nuclear polyhedrosis virus DNA (Pharmingen) and either pVL1392/KU-VP2, -VP4, -VP6, or -VP7 by the Lipofectin-mediated method. The baculovirus recombinants thus obtained were used for preparation of recombinant VP4, VLP-VP2/6, and VLP-VP2/6/7 in Tn5 cells.

Ab library. Abs were isolated from the Abs library called AIMS4, which was constructed in Y. Kurosawa's laboratory. In brief, B lymphocyte-rich fractions of human tissues such as tonsils, umbilical cord blood, peripheral blood, and bone marrow were used as gene sources of Abs (35; Y. Akahori et al., unpublished data). Using a phage-display system, the Fab form of an Ab fused to a truncated cp3 (Fab-cp3) was expressed on the phage surface. The library is composed of 10^{11} independently established clones, and it has been shown that >70% of the phages express Abs.

Screening of the library. Selection of phages exhibiting rotavirus (strain KU)-binding activity was performed by a panning method that was essentially the same as that described previously (20, 27). The immunotubes (Nunc-Immunomodules Polysorp) were coated with 200 μg of a purified KU virion/ml in phosphate-buffered saline containing 100 μg of Ca^{2+} and Mg^{2+}/ml [PBS(+)] overnight at 4°C. After a blocking step with 2% skim milk, a solution of phages (10^{14} CFU) was added to each tube, followed by incubation at room temperature for 2 h. The unbound clones were washed out four times with PBS(+). Bound phages were eluted with 0.1 M triethylamine (pH 12.3), and the eluent was then immediately neutralized with 1 M Tris-HCl (pH 6.8). *E. coli* DH12S cells cultured in 2xYT medium were infected with the eluted phages, precipitated by centrifugation, and then resuspended in 2xYT containing 1% glucose and 100 μg of ampicillin/ml, followed by superinfection with helper phages and further cultivation under kanamycin-selective conditions (70 μg/ml) in order to replicate phage clones harboring KU-reactive Abs. The phage clones obtained through this process were used for the next round of panning. The input titers of the phages and the number of washings with PBS(+) were 1.06×10^{13} and 8 for the second panning and 3.46×10^{13} and 16 for the third panning, respectively. After the third round of panning, DH12S cells infected with the selected phages were spread on LB plates containing 1% glucose and 100 μg of ampicillin/ml and incubated at 30°C overnight.

Preparation of various forms of Abs. The individual clones of *E. coli* infected with phages were grown in 2xYT medium containing 0.1% glucose and 100 μg of ampicillin/ml. After the addition of 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside), the Fab-cp3 molecules were initially accumulated in the periplasm of *E. coli* and then gradually secreted and/or released into the culture medium (crude Fab-cp3). On average, 1 μg of Fab-cp3 molecules/ml is present in the culture fluid. The Fab-cp3 molecules can be purified with anti-cp3 MAb-conjugated Sepharose beads. After isolation of phage particles, the gene encoding an Fab-cp3 molecule can be easily converted into another gene encoding an Fab-PP (P denotes a single Fc-binding domain of protein A) form of Ab by digestion with SalI followed by self-ligation (Fig. 1) (22). The Fab-PP molecules can be purified

TABLE 1. Oligonucleotide primers used for the conversion of phage antibodies (Fab-cp3) to human IgG1

Primer	Orientation[a]	Sequence
1-2HVH	F	5′-TTCCTCCTACTAGTGGCAGCTCCCAGATGGGTCCTGTCCCAGGTGCAGCTGGTGCAGTCTGG-3′
	R	5′-GGTGGAGGCACTCGAGACGGTGACCAGGGTTC-3′
1-2HVLCL	F	5′-CTACTCTGGCTCCGCGGTGCCAGACAGTCTGTGTTGACGCAGCCG-3′
	R	5′-TCGACTGGCGCGCCCTATGAACATTCTGTAGGGGCCACTGTCTTC-3′
2-3EVH	F	5′-TTCCTCCTACTAGTGGCAGCTCCCAGATGGGTCCTGTCCGAGGTGCAGCTGGTGGAGTCTGG-3′
	R	5′-GGTGGAGGCACTCGAGACGGTGACCATTGTCC-3′
2-3EVLCL	F	5′-CTACTCTGGCTCCGCGGTGCCAGAGAAACGACACTCACGCAGTCT-3′
	R	5′-TCGACTGGCGCGCCCTAACACTCTCCCCTGTTGAAGCTCTTTGTG-3′
2-11GVH	F	5′-TTCCTCCTACTAGTGGCAGCTCCCAGATGGGTCCTGTCCGAGGTGCAGCTGGTGGAGTCTGG-3′
	R	5′-GGTGGAGGCACTCGAGACGGTGACCGTGGTCC-3′
2-11GVLCL	F	5′-CTACTCTGGCTCCGCGGTGCCAGAGAAACGACACTCACGCAGTCT-3′
	R	5′-TCGACTGGCGCGCCCTAACACTCTCCCCTGTTGAAGCTCTTTGTG-3′

[a] F, forward; R, reverse. SpeI, XhoI, SacII, and AscI sites are underlined.

with an immunoglobulin G (IgG)-conjugated column. For conversion of an Fab to a human IgG1, the variable heavy-chain region (V_H) and the variable and constant light-chain region (V_L C_L) of an Fab fragment were amplified by PCR (15 cycles of amplification at 94°C for 2 min, 55°C for 2 min, and 72°C for 2 min) with primers designed for each clone (Table 1). After digestion with SpeI and XhoI for the V_H region and SacII and AscI for the V_L C_L region, the PCR products were subcloned into an IgG1 construction vector. The constructed IgG1 cassette was further cloned into the pCMV-Script expression vector (Fig. 1) (Akahori et al., unpublished). Expression of IgG1 molecules was performed by transfection of the IgG1 expression vectors into CHO-K1 cells by using GenePORTER (Gene Therapy Systems), which allows production and/or secretion of a whole human IgG1 type of Ab in culture fluid.

Enzyme-linked immunosorbent assay (ELISA). To determine the reactivity to or titer of the Abs for strain KU, immunoplates (96-well; Nalgen Nunc International) were coated with highly purified virions (500 ng/well) suspended in PBS(+) for 1 day at 4°C. After a blocking treatment with 5% bovine serum albumin in PBS(+), the plates were washed with PBS(+). For the reactivity study, the plates were then incubated with 50 ng of purified Fab-cp3 in PBS(+) at 4°C overnight. For the titration study, the plates were then incubated with serial dilutions of purified IgG1-formed Abs ranging from 0.01 to 5,000 ng/well in PBS(+) at 4°C overnight. In both cases, after the plates had been washed with PBS(+), a 1:2,500 dilution of peroxidase-conjugated goat anti-human IgG (H+L chain; MBL) was added to each well. One optical density at 492 nm (OD_{492}) unit was defined as the Ab concentration at which the OD_{492} reading was 1.0. To identify virus proteins recognized by the isolated Abs, the immunoplates were coated with 500 ng of purified Fab-cp3 at 4°C overnight, followed by blocking with 5% BSA in PBS(+). Then, unpurified VLPs or recombinant VP4 in the culture supernatant were added. After incubation at 4°C overnight and additional washing with PBS(+), 50 μl of 1:4,500 diluted rabbit anti-HRV antiserum (a mixture of 1:1,500 diluted rabbit anti-KU, anti-AK13, and anti-YO antiserum) was added to each well, followed by incubation at 4°C for 1 day. After the plates had been washed with PBS(+), a 1:2,500 dilution of peroxidase-conjugated goat anti-rabbit IgG (H+L chain; MBL) was added to each well. In all cases, the reactivity of the Abs to Ags was assessed after addition of the substrate.

Assay for virus-neutralizing activity. Screening of crude Fab-cp3 Abs for preliminary selection as to neutralizing activity and determination of the titers of the purified Abs against HRVs or antigenic mutants were determined by the fluorescent focus reduction (FF) method. A total of 25 μl of crude Fab-cp3 or purified Abs in PBS(+) at various concentrations (0.4 to 20 μg/ml) was mixed with an equal volume of virus suspension containing 3.6×10^4 to 14.4×10^4 focus-forming units in Eagle minimum essential medium, followed by reaction at 37°C for 1 h. Aliquots (50 μl) of the mixtures of Abs and viruses were inoculated onto MA104 cells in 96-well culture plates and, after an additional 1 h of incubation at 37°C, 100 μl of fresh Eagle minimum essential medium was added, followed by 16 to 18 h cultivation at 37°C. Fixation in the cold (−80°C), and reaction with the first and second Abs were performed as described previously (49). The neutralization assays were performed in duplicate and at least twice.

Sequence analysis. The nucleotide sequences of V_L and V_H regions were determined with an ABI Prism 320 genetic analyzer by using a BigDye terminator cycle sequencing kit (Applied Biosystems). The T7 primer (5′-TGTAATAC GACTCACTATAG-3′) and the huCH1J primer (5′-ATTAATAAGAGCTAT CCCGG-3′) were used for V_H and V_L sequencing, respectively.

Nucleotide sequence accession numbers. The nucleotide sequence data for the HRV neutralizing Abs reported in the present study have been submitted to the DDBJ database and were assigned the following accession numbers: AB114449 (for 1-2H H chain), AB114450 (2-1D H chain), AB114451 (2-2D H chain), AB114452 (2-3E H chain), AB114453 (2-4F H chain), AB114455 (2-7G H chain), AB114456 (2-9B H chain), AB114457 (2-9D H chain), AB114458 (2-11G H chain), AB114459 (2-12B H chain), AB114460 (3-1G H chain), AB114461 (4-3-C H chain), AB114461 (1-2H L chain), AB114462 (2-1D L chain), AB114463 (2-2D L chain), AB114464 (2-3E L chain), AB114465 (2-4F L chain), AB114466 (2-7G L chain), AB114467 (2-9B L chain), AB114468 (2-9D L chain), AB114469 (2-11G L chain), AB114470 (2-12B L chain), AB114471 (3-1G L chain), and AB114472 (4-3C L chain).

RESULTS

Isolation of Fab forms of Abs with neutralizing activities toward HRVs. After three rounds of panning, the recovered phages were used to infect *E. coli*, which was spread on plates containing ampicillin without infection with helper phages. We picked up 321 colonies and cultured them in 96-well plates. The supernatants, crude Fab-cp3, were directly subjected to analysis of neutralizing activities by means of the FF assay. Among the 321 clones analyzed, 24 appeared to exhibit neutralizing activities toward rotavirus strain KU.

Amino acid sequences of Fab H and L chains. In order to confirm the successful selection of phages with Fab-cp3 specific to strain KU and also to classify the 24 clones, we determined the sequences of variable regions of both their H and L chains. Some redundants were included in the 24 clones (Fig. 2), indicating the specific and successful selection and enrichment of KU-reactive phage Abs. The amino acid sequences of the H chains could be divided into 7 clones and the L chains could be divided into 16 clones (Fig. 2). As a result, 16 of the 24 clones were found to be independent.

Neutralizing activity of the purified Fab fragments. Since the use of crude Fab-cp3 in the FF assay gave ambiguous

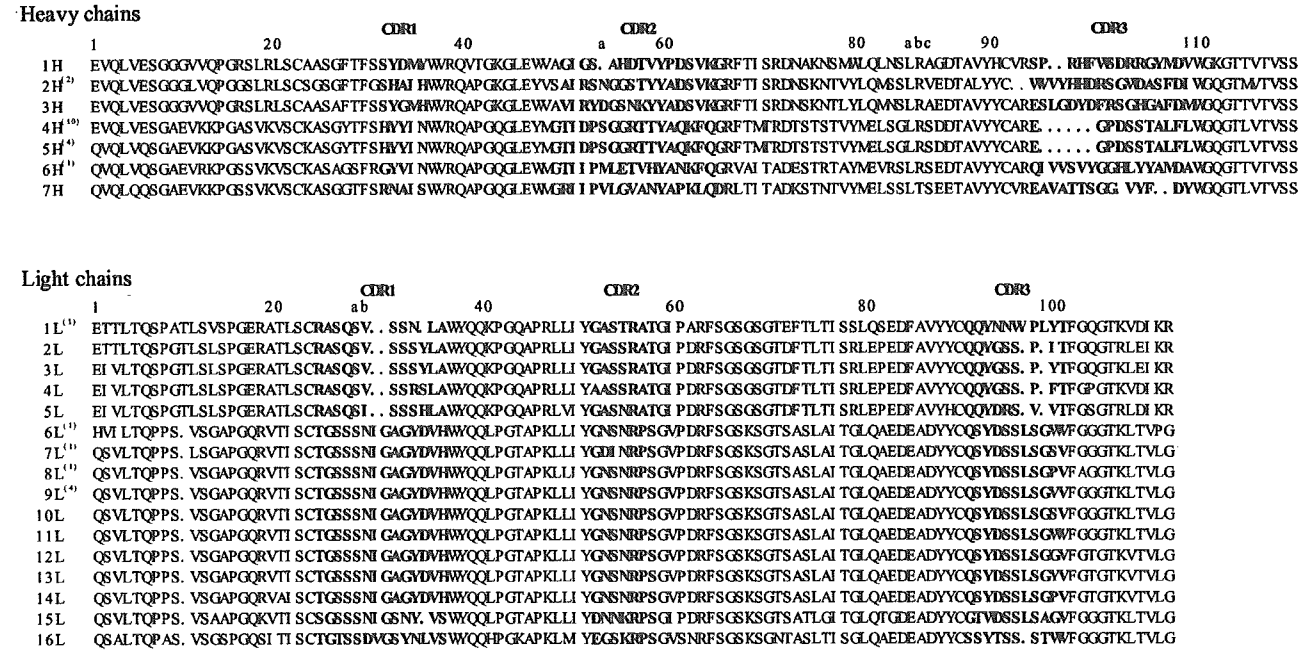

FIG. 2. Amino acid sequences of variable regions of the H and L chains of Abs that appeared to exhibit neutralizing activities toward strain KU. The sequences of CDRs are shown in boldface. Numbers in parentheses indicate number of redundant clones found among the 24 Abs analyzed, with 16 Abs being independent. The combinations of H and L chains in the independent Abs are indicated in Table 2. The numbering of amino acid positions is according to the method of Kabat et al. (23).

results due to contaminants in the *E. coli* culture supernatant, the Fab-cp3 coding phagemid DNAs of the 16 independent clones (2-7G, 3-1G, 2-9D, 2-4F, 2-9B, 1-2H, 4-3C, 2-12B, 2-2D, 2-3E, 2-1D, 2-11G, 1-8A, 2-2G, 2-5G, and 1-4D) were reconstructed to produce Fab-PP fragments (Fig. 1) to facilitate purification. After purification by means of affinity selection, their neutralizing activities toward strain KU were assessed by FF assay. Except for 1-4D, 1-8A, 2-2G, and 2-5G, we could detect the neutralizing activity against strain KU (Table 2). Although the Ag-binding site of each Ab is formed by amino acid residues in the six complementarity-determining regions (CDRs) of the H and L chains, the contribution of CDR3 of the H chain to Ag specificity is greatest among them in a usual case, especially that to protein Ags (21). Judging from this, the two H chains, 4H and 5H, are essentially the same (Fig. 2), and the 12 clones exhibiting the neutralizing activities toward KU could be classified into three groups (Table 2). We finally selected 1-2H, 2-3E, and 2-11G as representative clones of the three group, and further investigations were carried out on these three.

Virus proteins recognized by Abs. The reactivity of the three representative Fab fragments was examined by means of ELISA, their specific reactivity with strain KU being shown (Fig. 3A). Although an isolated VP4 molecule could expose its neutralizing epitope on the surface, VP7 exposed its epitope only when the molecule was embedded in inner proteins VP2 and VP6 (49). Therefore, we prepared two kinds of VLP, VLP-V2/6 and VLP-2/6/7, and recombinant VP4. As shown in Fig. 3B, the targeted virus protein of 1-2H and 2-3E was found to be VP4, and that of 2-11G was found to be VP7. A Western blot analysis to confirm the ELISA results was unsuccessful (data not shown).

Neutralizing activities toward various HRV strains. After conversion of the Fab form into an IgG1 Ab, we analyzed the neutralizing activities of the three Abs against 13 HRVs by means of the FF assay (Table 3). The 1-2H Ab neutralized 7 strains—S2, L26, KU, Wa, YO, Hosokawa, and WI61—all of which exhibited either P[4] or P[8] type specificity on VP4. The 2-3E Ab showed neutralizing activities toward 8 strains—M37,

TABLE 2. Neutralization activity of Fab-PP forms of Abs to strain KU

Clone (n)[a]	H chain	L chain	Antibody concn (μg/ml)[b]
2-7G (1)	4H	6L	1.6
3-1G (1)		7L	8.0
2-9D (4)		9L	1.6
2-4F		10L	1.6
2-9B		12L	1.6
1-2H	5H	13L	1.6
4-3C (1)		8L	1.6
2-12B		11L	1.6
2-2D		14L	1.6
2-3E (1)	2H	1L	1.6
2-1D		15L	1.6
2-11G	1H	2L	1.6
1-8A	3H	5L	>40.0
2-2G	6H	4L	>40.0
2-5G		3L	>40.0
1-4D	7H	16L	>40.0

[a] n = number of redundant clones found among the 24 clones analyzed.
[b] Concentration of antibodies required for a 50% reduction of the fluorescent focus in the FF assay.

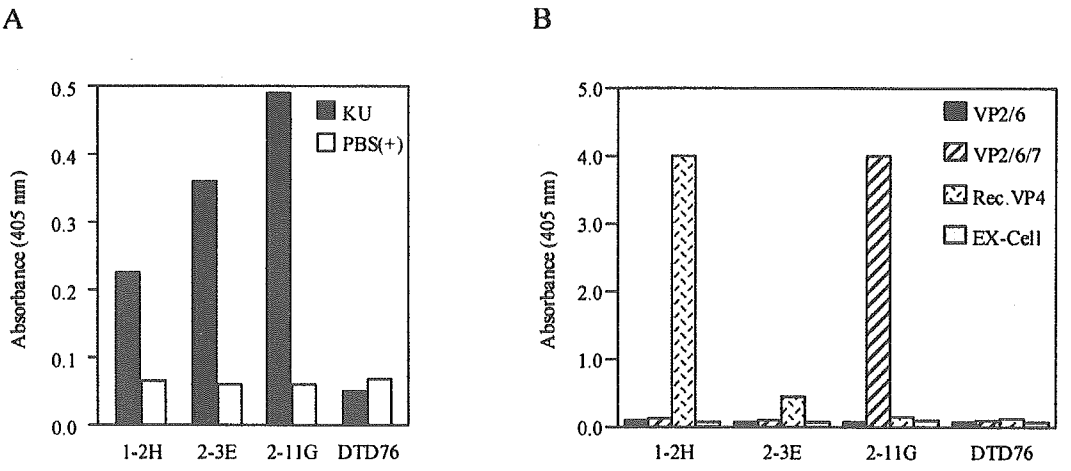

FIG. 3. ELISA of the three Abs (purified Fab-cp3 forms). (A) Reactivities of the three Abs to strain KU. Purified virions suspended in PBS(+) were used as Ags. (B) Virus proteins directed by the three Abs. The culture fluids of Tn5 cells containing VLP-VP2/6, VLP-VP2/6/7, or recombinant VP4 were used as Ags. EX-Cell was the culture medium of Tn5. DTD76 is anti-diphtheria toxin Ab isolated from AIMS4, which was used as a negative control. VP2/6, VLPs constructed with VP2 and VP6; VP2/6/7, VLPs constructed with VP2, VP6, and VP7; Rec.VP4, recombinant VP4.

1076, McN13, KU, Wa, YO, Hosokawa, and WI61—all of which are classified as either a P[6] or P[8] virus strain. The 2-11G Ab reacted with four strains—M37, KU, Wa, and K8—all of which are G1 strains. The conclusion that the 2-11G Ab reacts with VP7 with the G1 serotype was further supported by using two VP7 single gene reassortants, UK/Wa and UK/DS1. The former has VP7 of G1 and VP4 of P[5] specificity, and the latter has VP7 of G2 and VP4 of P[5] specificity. The 2-11G Ab neutralized only the former strain. Of the 13 HRVs examined, 11 were neutralized by any of the three Abs. The concentration of Abs required for 50% neutralization ranged from 0.8 to 20 μg/ml, which corresponds to 5.3 to 133 nM.

Neutralizing activities toward antigenic mutants. We also examined the neutralizing activities of the three representative Abs isolated in the present study with the mouse MAb-resistant mutants prepared in our previous studies (45–47). All of the three Abs of the Fab-cp3 form turned out to neutralize all of the mutants examined (Table 4). The concentration of the each Ab required for 50% reduction of the fluorescent focus in the FF assay was <1.6 μg/ml.

Titers of the Abs to strain KU. We determined the titers of three Abs (IgG1 form) to strain KU by ELISA (Fig. 4). All three Abs showed dose-dependent binding. There was no correlation between the absorbance density and the neutralizing activity of each Ab. The values for one OD_{492} unit for the 1-2H, 2-3E, and 2-11G Abs were 0.70, 0.75, and 1.8 μg/ml, respectively.

DISCUSSION

Ab libraries constructed with a means of a phage-display system are convenient for the rapid isolation of Abs specific for various Ags (2, 27, 31, 44, 52). Recently, several recombinant human Fab fragments exhibiting neutralizing activity toward viruses such as human immunodeficiency virus types 1 and 2, Ebola virus, measles virus, Puumala virus, and respiratory syncytial virus were prepared by means of a phage display system (1, 3, 6, 7, 28, 37, 50). Since the amount of surface proteins with neutralization epitopes on viruses is small and the immunogenicity of inner proteins is quite high, it is generally much more difficult to obtain MAbs with neutralizing capacity than non-neutralizing MAbs. This requires some modifications of the panning and/or screening processes. For example, blockade of a common, nonneutralizing epitope with a representative Fab has been used in panning assays to isolate respiratory syncytial virus-neutralizing human MAbs (50). In the present study, we used highly purified virion for panning and directly screened numerous clones by means of rapid microneutralization FF tests, which have been found to be very efficient for screening neutralizing MAbs with common mouse hybridoma technology (49).

We used HRV strain KU as the Ag for panning, since strain KU exhibits representative G1 and P[8] specificity, which is the

TABLE 3. Neutralization of HRVs and reassortants by purified human IgG1s

Strain	P genotype	G serotype	Antibody concn (μg/ml)[a]		
			1-2H	2-3E	2-11G
S2	P[4]	G2	4–20	>20	>20
L26	P[4]	G12	0.8	>20	>20
M37	P[6]	G1	>20	4	0.8
1076	P[6]	G2	>20	4	>20
McN13	P[6]	G3	>20	4	>20
KU	P[8]	G1	8	1.6	0.4
Wa	P[8]	G1	4	4	4–20
YO	P[8]	G3	8	1.6	>8
Hosokawa	P[8]	G4	4	0.8	>20
WI61	P[8]	G9	4–20	0.8–4	>20
AU-1	P[9]	G3	>20	>20	>20
K8	P[9]	G1	>20	>20	4
69M	P[10]	G8	>20	>20	>20
UK/Wa	P[5]	G1	>20	>20	4
UK/DS-1	P[5]	G2	>20	>20	>20

[a] Antibody concentration that reduced the fluorescent focus count by >50% in the neutralization test (FF assay).

TABLE 4. Neutralization activity of purified antibodies (cp3 form) to antigenic variants

Strain	Mutant protein	No. of virus-infected cells with:[a]											
		1-2H at:			2-3E at:			2-11G at:			DTD76 at:		
		1.6 μg/ml	8 μg/ml	40 μg/ml	1.6 μg/ml	8 μg/ml	40 μg/ml	1.6 μg/ml	8 μg/ml	40 μg/ml	1.6 μg/ml	8 μg/ml	40 μg/ml
V-1E6	VP4	63	69	60	4	2	1	14	2	14	296	378	358
V-1F2	VP4	64	76	43	45	108	21	10	5	1	350	462	368
V-1S3	VP4	31	32	19	127	91	18	26	9	4	312	302	304
V-2C2	VP4	87	46	56	9	3	4	16	0	2	296	342	344
V-4D7	VP4	68	13	12	6	1	0	35	3	4	396	222	354
V-6B11	VP4	21	17	11	8	63	4	3	1	0	212	324	240
V-3C7	VP7	121	123	81	14	17	11	19	3	2	482	532	554
V-4C2	VP7	96	87	120	20	9	5	23	2	3	596	660	656
V-5H1	VP7	146	156	90	13	4	4	46	1	1	372	395	355
V-6A11	VP7	57	84	79	57	23	27	24	8	6	425	554	564
V-KU4	VP7	47	22	14	3	4	0	6	0	3	206	186	206
KU	–	26	15	13	3	3	3	52	3	5	282	300	305

[a] That is, the numbers of virus-infected cells detected by the FF method (see Materials and Methods). Virus-positive cells in a one-ninth area of one well of a 96-well tissue culture plate were counted. Results are presented as the means for two independent experiments performed in duplicate.

most prevalent HRV serotype worldwide (24), and since the neutralization epitopes on VP4 and VP7 of the strain have well been characterized by using mouse MAbs (36, 45, 47, 49). The three human Abs characterized (1-2H, 2-3E, and 2-11G) are specific to P[8], P[4]; P[8], P[6]; and G1 HRVs, respectively. In particular, the former two are broadly reactive with a wide spectrum of HRVs. Since a total of 15 G serotypes have been defined for rotavirus and at least 10 G serotypes have been isolated from humans, it is desirable to prepare such broadly reactive heterotypic Abs for therapeutic purposes. The reactivity of the three Abs covered most HRV strains, and they indeed neutralized 11 of the 13 HRVs examined. In previous studies, an Ab response to cross-reactive neutralization epitopes (YO-2C2 epitopes) was observed much more frequently in schoolchildren and adults than in infants (13, 48). Since the library was constructed from the tissues of adults, who would have been repeatedly infected with HRVs with distinct serotypes and would have immunological memory for cross-reactive neutralization epitopes, cross-reactive Abs may have been readily selected in the present study. In other words, a cross-reactive immune response should be common in the immune system in humans, particularly in adults, infected with rotaviruses.

A number of murine MAbs have been prepared for rotaviruses by means of conventional hybridoma technology. Although many of them were directed to the inner protein VP6,

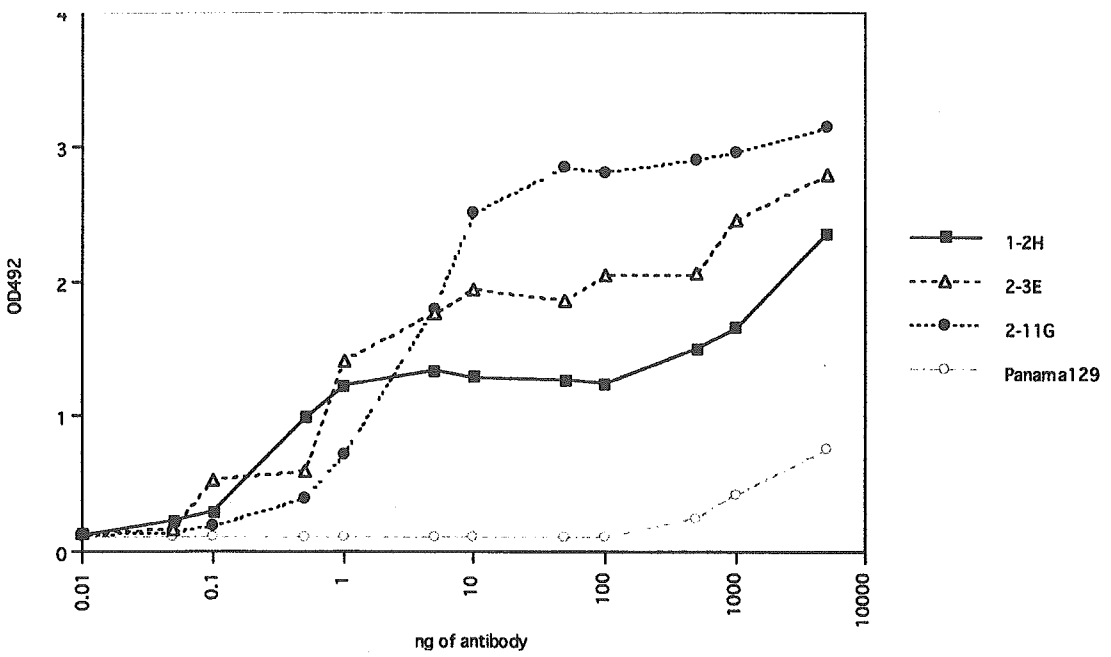

FIG. 4. Abs titration curves on ELISA. The reactivities between purified IgG1-formed Abs and purified virions of strain KU were assessed. The assay was performed in duplicate, and the mean data are plotted. Panama129 is the IgG1-formed anti-influenza virus Ab isolated from the AIMS4 library and converted to the IgG1 form as described in Materials and Methods.

some were directed to VP7 and VP4. However, the number of neutralizing MAbs with hetetrotypic specificity was limited. In particular, ones directed to HRVs were few; i.e., there was only one to VP7 and seven to VP4 (26, 36, 45–47, 49). They have been useful for the analysis of heterotypic neutralization epitopes on VP7 and VP4. By analyzing the mutants resistant to each of the neutralizing mouse MAbs directed to VP4 or VP7 of HRVs, critical amino acids in the neutralization epitopes have been identified (29, 45, 46). It has been shown that the cross-reactive neutralization epitopes on VP4 are the 305th, 385th, 392nd, 428th, 433rd, and 439th amino acid residues (26, 46, 47) and that the G1-specific epitopes on VP7 are the 94th and 96th residues (45). Furthermore, by means of neutralization tests on various combinations of MAb-resistant mutants and MAbs, operational maps of the neutralization epitopes have been constructed (26, 36). We examined the reactivities of the three human Abs isolated in the present study with the MAb-resistant mutants prepared in our previous studies (45–47): mutants resistant to anti-VP4 MAbs (YO-2C2, KU-6B11, YO-1S3, ST-1F2, KU-4D7, and YO-1E6) and mutants resistant to anti-VP7 MAbs (KU-2, KU-4, KU-3C7, KU5H1, KU-6A11, and YO-4C2). These three Abs turned out to neutralize all of the mutants examined (Table 4). This finding strongly suggests that the human Abs isolated in the present study recognize neutralization epitopes distinct from those recognized by mouse MAbs obtained to date. These results could have been predicted, since the specificity showed by the human Abs, such as 1-2H Ab to P[4] and P[8] and 2-3E Ab to P[6] and P[8], had not been shown by mouse MAbs isolated to date. These results imply that the cross-reactive neutralization epitopes recognized by humans, in particular adults, infected with rotaviruses and by mice immunized with rotaviruses are quite distinct. We are now attempting to prepare mutants resistant to each human Ab for analysis of the neutralization epitopes recognized by them.

The mechanism of protective immunity against rotavirus infection has not been well elucidated. Both humoral and cellular immunity are likely to be involved in the protection from rotavirus infection (11, 24, 40). The mucosal Ab response has been believed to be effective for such protection. Furthermore, passive immunity has also been found to be effective (40). Maternal transfer of anti-rotavirus immunoglobulins protects babies from rotavirus infection. Oral administration of bovine immunoglobulins, mouse MAbs, and human immunoglobulins has been found to be effective for protecting suckling mice from rotavirus infection (9, 30, 41). In addition, therapeutic reports on the passive immunity of children with rotavirus diarrhea have also been published. Guarino et al. reported that the oral administration of human serum immunoglobulins to children with rotavirus-induced diarrhea resulted in a faster recovery from the disease (15), even though the children were immunocompromised due to human immunodeficiency virus infection (16). In contrast, there have also been reports showing no clinical effect of oral administration of bovine immunoglobulins for prophylaxis or therapy for HRV infection (4, 10). We are now examining a mouse model to determine whether the human MAbs prepared in the present study are effective and practically relevant to immunotherapy and/or prophylaxis for diseases caused by rotavirus.

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REFERENCES

1. **Barbas, C. F., III, E. Bjorling, F. Chiodi, N. Dunlop, D. Cababa, T. M. Jones, S. L. Zebedee, M. A. A. Persson, P. L. Nara, E. Norrby, and D. R. Burton.** 1992. Recombinant human Fab fragments neutralize human type 1 immunodeficiency virus in vitro. Proc. Natl. Acad. Sci. USA **89:**9339–9343.
2. **Barbas, C. F., III, A. S. Kang, R. A. Lerner, and S. J. Benkovic.** 1991. Assembly of combinational antibody libraries on phage surfaces: the gene III site. Proc. Natl. Acad. Sci. USA **88:**7978–7982.
3. **Björling, E., E. von Garrelts, A. Morner, M. Ehnlund, and M. A. A. Persson.** 1999. Human neutralizing immunodeficiency virus type 2-specific Fab molecules generated by phage display. J. Gen. Virol. **80:**1987–1993.
4. **Brunser, O., J. Espinoza, G. Figueroa, M. Araya, E. Spencer, H. Hilpert, H. Link-Amster, and H. Brussow.** 1992. Field trial of an infant formula containing anti-rotavirus and anti-*Escherichia coli* milk antibodies from hyperimmunized cows. J. Pediatr. Gastroenterol. Nutr. **15:**63–72.
5. **Cale, C. M., and N. J. Klein.** 2002. The link between rotavirus vaccination and intussusception: implications for vaccine strategies. Gut **50:**11–12.
6. **de Carvalho Nicacio, C., Å. Lundkvist, K. B. Sjölander, A. Plyusnin, E. M. Salonen, and E. Björling.** 2000. A neutralizing recombinant human antibody Fab fragment against Puumala hantavirus. J. Med. Virol. **60:**446–454.
7. **de Carvalho Nicacio, C., R. A. Williamson, P. W. H. I. Parren, Å. Lundkvist, D. R. Burton, and E. Björling.** 2002. Neutralizing human Fab fragments against measles virus recovered by phage display. J. Virol. **76:**251–258.
8. **Desselberger, U., M. Iturriza-Gómara, and J. J. Gray.** 2000. Rotavorus epidemiology and surveillance. Novartis Found. Symp. **238:**125–152.
9. **Ebina, T., A. Sato, K. Umezu, N. Ishida, S. Ohyama, A. Ohizumi, K. Aikawa, S. Katagiri, N. Katsushima, and A. Imai.** 1983. Prevention of rotavirus infection by cow colostrum antibody against human rotaviruses. Lancet **ii:** 1029–1030.
10. **Ebina, T., A. Sato, K. Umezu, N. Ishida, S. Ohyama, A. Oizumi, K. Aikawa, S. Katagiri, N. Katsushima, and A. Imai.** 1985. Prevention of rotavirus infection by oral administration of cow colostrum containing anti-human rotavirus antibody. Med. Microbiol. Immunol. **174:**177–185.
11. **Estes, M. K.** 2001. Rotaviruses and their replication, p. 1747–1786. *In* D. M. Knipe and P. M. Howley (ed.), Fields virology, 4th ed. Lippincott/The Williams & Wilkins Co., Philadelphia, Pa.
12. **Gilger, M. A., D. O. Matson, M. E. Conner, H. M. Rosenblatt, M. J. Finegold, and M. K. Estes.** 1992. Extraintestinal rotavirus infections in children with immunodeficiency. J. Pediatr. **120:**912–917.
13. **Green, K. Y., K. Taniguchi, E. R. Mackow, and A. Z. Kapikian.** 1990. Homotypic and heterotypic epitope-specific antibody responses in adult and infant rotavirus vaccines: implications for vaccine development. J. Infect. Dis. **161:**667–679.
14. **Greenberg, H. B., J. Valdesuso, K. van Wyke, K. Midthun, M. Walsh, V. McAuliffe, R. G. Wyatt, A. R. Kalica, J. Flores, and Y. Hoshino.** 1983. Production and preliminary characterization of monoclonal antibodies directed at two surface proteins of rhesus rotavirus. J. Virol. **47:**267–275.
15. **Guarino, A., R. B. Canani, S. Russo, F. Albano, M. B. Canani, F. M. Ruggeri, G. Donelli, and A. Rubino.** 1994. Oral immunoglobulins for treatment of acute rotaviral gastroenteritis. Pediatrics **93:**12–16.
16. **Guarino, A., S. Russo, A. Castaldo, M. I. Spagnuolo, L. Tarallo, and A. Rubino.** 1996. Passive immunotherapy for rotavirus-induced diarrhoea in children with HIV infection. AIDS **10:**1176–1178.
17. **Hammarström, L.** 1999. Passive immunity against rotavirus in infants. Acta Pediatr. Suppl. **430:**127–132.
18. **Hoshino, Y., L. J. Saif, M. M. Sereno, R. M. Chanock, and A. Z. Kapikian.** 1988. Infection immunity of piglets to either VP3 or VP7 outer capsid protein confers resistance to challenge with a virulent rotavirus bearing the corresponding antigen. J. Virol. **62:**744–748.
19. **Hoshino, Y., M. M. Sereno, K. Midthun, J. Flores, A. Z. Kapikian, and R. M. Chanock.** 1985. Independent segregation of two antigenic specificities (VP3 and VP7) involved in neutralization of rotavirus infectivity. Proc. Natl. Acad. Sci. USA **82:**8701–8704.
20. **Iba, Y., W. Ito, and Y. Kurosawa.** 1997. Expression vectors for the introduction of highly diverged sequences into the six complementarity-determining regions of an antibody. Gene **194:**35–46.
21. **Ichihara, Y., H. Matsuoka, and Y. Kurosawa.** 1988. Organization of human immunoglobulin heavy chain diversity gene loci. EMBO J. **7:**4141–4150.
22. **Ito, W., and Y. Kurosawa.** 1993. Development of an artificial antibody system with multiple valency using an Fv fragment fused to a fragment of protein A. J. Biol. Chem. **268:**20668–20675.

23. **Kabat, E. A., T. T. Wu, H. M. Perry, K. S. Gottesman, and C. Foeller.** 1991. Sequences of proteins of immunological interest. National Institutes of Health, Bethesda, Md.
24. **Kapikian, A. Z., Y. Hoshino, and R. M. Chanock.** 2001. Rotaviruses, p. 1787–1833. *In* D. M. Knipe and P. M. Howley (ed.), Fields virology, 4th ed. Lippincott/The Williams & Wilkins Co., Philadelphia, Pa.
25. **Kapikian, A. Z., Y. Hoshino, R. M. Chanock, and I. Pérez-Schael.** 1996. Efficacy of a quadrivalent rhesus rotavirus-based human rotavirus vaccine aimed at preventing severe rotavirus diarrhea in infants and young children. J. Infect. Dis. **174**(Suppl. 1):S65–S72.
26. **Kobayashi, N., K. Taniguchi, and S. Urasawa.** 1990. Identification of operationally overlapping and independent cross-reactive neutralization regions on human rotavirus VP4. J. Gen. Virol. **71**:2615–2623.
27. **Marks, J. D., H. R. Hoogenboom, T. P. Bonnert, J. McCafferty, A. D. Griffiths, and G. Winter.** 1991. By-passing immunization. Human antibodies from V-gene libraries displayed on phage. J. Mol. Biol. **222**:581–597.
28. **Maruyama, T., L. L. Rodroguez, P. B. Jahring, A. Sanchez, A. S. Khan, S. T. Nichol, C. J. Peters, P. W. H. I. Parren, and D. R. Burton.** 1999. Ebola virus can be effectively neutralized by antibody produced in natural human infection. J. Virol. **73**:6024–6030.
29. **Matsui, S. M., E. R. Mackow, and H. B. Greenberg.** 1989. Molecular determinant of rotavirus neutralization and protection. Adv. Virus Res. **36**:181–214.
30. **Matsui, S. M., P. A. Offit, P. T. Vo, E. R. Mackow, D. A. Benfield, R. D. Shaw, L. Padilla-Noriega, and H. B. Greenberg.** 1989. Passive protection against rotavirus-induced diarrhea by monoclonal antibodies to the heterotypic neutralization domain of VP7 and the VP8 fragment of VP4. J. Clin. Microbiol. **27**:780–782.
31. **McCafferty, J., A. D. Griffiths, G. Winter, and D. J. Chiswell.** 1990. Phage antibodies: filamentous phage displaying antibody variable domains. Nature **348**:552–554.
32. **Midthun, K., Y. Hoshino, A. Z. Kapikian, and R. M. Chanock.** 1986. Single gene substitution rotavirus reassortants containing the major neutralization protein (VP7) of human rotavirus serotype 4. J. Clin. Microbiol. **24**:822–826.
33. **Miller, M. A., and L. McCann.** 2000. Policy analysis of the use of hepatitis B, *Haemophilus influenzae* type B-, *Streptococcus pneumoniae*-conjugate, and rotavirus vaccines in national immunization schedules. Health Econ. **9**:19–35.
34. **Mori, I., K. Matsumoto, K. Sugimoto, M. Kimura, N. Daimon, T. Yokochi, and Y. Kimura.** 2002. Prolonged shedding of rotavirus in a geriatric inpatient. J. Med. Virol. **67**:613–615.
35. **Morino, K., H. Katsumi, Y. Akahori, Y. Iba, M. Shinohara, Y. Ukai, Y. Kohara, and Y. Kurosawa.** 2001. Antibody fusions with fluorescent proteins: a versatile reagent for profiling protein expression. J. Immunol. Methods **257**:175–184.
36. **Morita, Y., K. Taniguchi, T. Urasawa, and S. Urasawa.** 1988. Analysis of serotype-specific neutralization epitopes on VP7 of human rotavirus by the use of neutralizing monoclonal antibodies and antigenic variants. J. Gen. Virol. **69**:451–458.
37. **Moulard, M., S. K. Phogat, Y. Shu, A. F. Labrijn, X. Xiao, J. M. Binley, M. Zhang, I. A. Sidorov, C. C. Broder, J. Robinson, P. W. H. I. Parren, D. R. Burton, and D. S. Dimitrov.** 2002. Broadly cross-reactive HIV-1-neutralizing human monoclonal Fab selected for binding to gp120-CD4-CCR5 complexes. Proc. Natl. Acad. Sci. USA **99**:6913–6918.
38. **Murphy, T. V., P. M. Gargiullo, M. S. Massoudi, D. B. Nelson, A. O. Jumaan, C. A. Okoro, L. R. Zanardi, S. Setia, E. Fair, C. W. LeBaron, M. Wharton, and J. R. Livingood.** 2001. For the rotavirus intussusception investigation team: intussusception among infants given an oral rotavirus vaccine. N. Engl. J. Med. **344**:564–572.
39. **Nakajima, H., T. Nakagomi, T. Kamisawa, N. Sakaki, K. Muramoto, T. Mikami, H. Nara, and O. Nakagomi.** 2001. Winter seasonality and rotavirus diarrhoea in adults. Lancet **357**:1950.
40. **Offit, P. A.** 1996. Host factors associated with protection against rotavirus disease: the skies are clearing. J. Infect. Dis. **174**(Suppl. 1):S59–S64.
41. **Offit, P. A., R. D. Shaw, and H. B. Greenberg.** 1986. Passive protection against rotavirus-induced diarrhea by monoclonal antibodies to surface proteins VP3 and VP7. J. Virol. **58**:700–703.
42. **Pedley, S., F. Hundley, I. Chrystie, M. A. McCrae, and U. Desselberger.** 1984. The genomes of rotaviruses isolated from chronically infected immunodeficient children. J. Gen. Virol. **65**:1141–1150.
43. **Peigue-Lafeuille, H., C. Henquell, M. Chambon, N. Gazuy, C. De Champs, and R. Cluzel.** 1991. Nosocomial rotavirus infections in adult renal transplant recipients. J. Hosp. Infect. **18**:67–70.
44. **Sheets, M. D., P. Amersdorfer, R. Finnern, P. Sargent, E. Lindquist, R. Schier, G. Hemingsen, C. Wong, J. C. Gerhart, and J. D. Marks.** 1998. Efficient construction of a large nonimmune phage antibody library: the production of high-affinity human single-chain antibodies to protein antigens. Proc. Natl. Acad. Sci. USA **95**:6157–6162.
45. **Taniguchi, K., Y. Hoshino, K. Nishikawa, K. Y. Green, W. L. Maloy, Y. Morita, S. Urasawa, A. Z. Kapikian, R. M. Chanock, and M. Gorziglia.** 1988. Cross-reactive and serotype-specific neutralization epitopes on VP7 of human rotavirus: nucleotide sequence analysis of antigenic mutants selected with monoclonal antibodies. J. Virol. **62**:1870–1874.
46. **Taniguchi, K., W. L. Maloy, K. Nishikawa, K. Y. Green, Y. Hoshino, S. Urasawa, A. Z. Kapikian, R. M. Chanock, and M. Gorziglia.** 1988. Identification of cross-reactive and serotype 2-specific neutralization epitopes on VP3 of human rotavirus. J. Virol. **62**:2421–2426.
47. **Taniguchi, K., Y. Morita, T. Urasawa, and S. Urasawa.** 1987. Cross-reactive neutralization epitopes on VP3 of human rotavirus: analysis with monoclonal antibodies and antigenic variants. J. Virol. **61**:1726–1730.
48. **Taniguchi, K., T. Urasawa, N. Kobayashi, M. U. Ahmed, N. Adachi, S. Chiba, and S. Urasawa.** 1991. Antibody response to serotype-specific and cross-reactive neutralization epitopes on VP4 and VP7 after rotavirus infection or vaccination. J. Clin. Microbiol. **29**:483–487.
49. **Taniguchi, K., S. Urasawa, and T. Urasawa.** 1985. Preparation and characterization of neutralizing monoclonal antibodies with different reactivity patterns to human rotaviruses. J. Gen. Virol. **66**:1045–1053.
50. **Tsui, P., M. A. Tornetta, R. S. Ames, B. C. Bankosky, S. Griego, C. Silverman, T. Porter, G. Moore, and R. W. Sweet.** 1996. Isolation of a neutralizing human RSV antibody from a dominant, non-neutralizing immune repertoire by epitope-blocked panning. J. Immunol. **157**:772–780.
51. **Ward, R. L., D. R. Knowlton, G. M. Schiff, Y. Hoshino, and H. B. Greenberg.** 1988. Relative concentrations of serum neutralizing antibody to VP3 and VP7 proteins in adults infected with a human rotavirus. J. Virol. **62**:1543–1549.
52. **Winter, G., A. D. Griffiths, R. E. Hawkins, and H. R. Hoogenboom.** 1994. Making antibodies by phage display technology. Annu. Rev. Immunol. **12**: 433–455.

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Isolation and characterization of a new species of kobuvirus associated with cattle

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A cytopathic agent was isolated using Vero cells from the culture medium of HeLa cells that had been used for more than 30 years in our laboratory. This agent, termed U-1 strain, was serially passed in Vero cells with distinct CPE. Particles of U-1 strain negatively stained with phosphotungstic acid exhibited a distinct surface that resembled Aichi virus. The RNA genome of U-1 strain comprises 8374 nt, with a genome organization analogous to that of picornaviruses. Possible cleavage sites of the large ORF, which encoded a leader protein prior to the capsid protein region, were assigned following amino acid alignment with Aichi virus. The virus sequence had 33 and 75 % amino acid identity with the Aichi virus VP1 and 3D regions, respectively, but no more than 23 and 36 % with those of the prototype strains of other *Picornaviridae*. The dendrogram based on the P1, P2 and P3 proteins indicated that U-1 strain is genetically included in the genus *Kobuvirus* but is distinct from Aichi virus. Of 72 cattle sera, 43 (59·7 %) were positive for neutralizing antibody against U-1 strain at a titre of 1 : 8 or more. However, sera from 190 humans, 242 monkeys, 139 pigs, 5 horses, 22 dogs and 9 cats did not neutralize U-1 strain at a 1 : 4 dilution. RNA corresponding to U-1 strain was detected in 12 (16·7 %) of 72 faecal samples from cattle by RT-PCR. These results indicated that U-1 strain, suspected to be a contaminant from calf sera, is a new species of the genus *Kobuvirus*, now termed bovine kobuvirus.

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INTRODUCTION

Picornaviruses consist of a naked capsid that surrounds a core of ssRNA. Hydrated native particles are 30 nm in diameter but vary from 22 to 30 nm in electron micrographs, which reveal the virion as a smooth sphere. *Picornaviridae* comprises nine genera: *Enterovirus*, *Rhinovirus*, *Aphthovirus*, *Cardiovirus*, *Hepatovirus*, *Parechovirus*, *Erbovirus*, *Kobuvirus* and *Teschovirus* (King *et al.*, 2000; Pringle, 1999). Aichi virus is a species of the genus *Kobuvirus* (Stanway *et al.*, 2002), with a rough virion surface, and has been isolated from patients with gastroenteritis (Yamashita *et al.*, 1991). The Aichi virus (A846/88) genome is 8280 nt long and encodes a single polyprotein of 2432 aa. This polyprotein has a clear similarity with other picornaviruses. However, there are some characteristic regions of the Aichi virus genome organization. For example, Aichi virus has an L protein consisting of 170 aa and only three structural proteins, VP0, VP3 and VP1 (Yamashita *et al.*, 1998). Hughes & Stanway (2000) observed conserved motifs, previously unrecognized, in the Aichi virus, human parechovirus (HPeV) and avian encephalomyelitis virus (AEV) 2A proteins.

The GenBank accession numbers of the sequences reported in this paper are AB084788 and AB097152–AB097166.

Recently, the genome sequences of AEV and Ljungan virus from bank voles have been determined and revealed to be members of the *Picornaviridae* and most closely related to hepatitis A virus and HPeV, respectively (Marvil *et al.*, 1999; Niklasson *et al.*, 1999). Thus, most picornavirus genera consist of two or more species. We have studied 17 virus isolates of human patients with gastroenteritis and determined these to be Aichi viruses via a neutralization test with Aichi virus (A846/88) antisera (Yamashita *et al.*, 1991, 1993, 1995). To date, Aichi virus is the only species of the genus *Kobuvirus*.

Using Vero cells, we detected recently a cytopathic agent in the culture medium of HeLa cells. This agent could not be neutralized by Aichi virus antisera but contained some features similar to Aichi virus and reacted with antibody raised to Aichi virus by ELISA. In this study, we demonstrate by sequencing the entire genome that this agent is a new species of the genus *Kobuvirus*. Moreover, an epidemiological study performed suggested that our HeLa cells were contaminated with this virus, termed U-1 strain, which had originated presumably from cattle sera.

METHODS

HeLa cells. HeLa cells were provided to our laboratory by H. Sunaga (Nagoya University School of Medicine, Nagoya, Japan)

in the 1960s. The unique colony name is not available. At that time, HeLa cells were cultivated using MEM containing 10 % calf serum. From the 1980s, however, foetal calf serum (FCS) was used for the medium instead of calf serum. HeLa cl.2 cells (ATCC) were purchased from Dainippon Seiyaku and cultivated in plastic flasks using MEM with 10 % FCS for virus isolation tests.

Isolation of U-1 strain and preparation of antisera. Medium from cultivated HeLa cells was inoculated onto Vero cells. CPE was confirmed only in the sample medium cultivated with the HeLa cells provided from Nagoya University. The cytopathogenic agent was plaque-cloned and termed U-1 strain. Serological, biochemical and biophysical analyses were performed with U-1 strain, as described previously (Yamashita *et al.*, 1991). Briefly, U-1 strain was incubated in 10 % chloroform for 10 min at room temperature and in MEM (pH 3·5) for 3 h at room temperature to evaluate its stability in organic solvents and under acidic conditions, respectively. The type of U-1 strain nucleic acid was determined by examining the effects of $10^{-4.5}$ M 5-iodo-2′-deoxyuridine (IUDR), a DNA virus inhibitor of virus replication. U-1 strain cultivated in Vero cells was purified using caesium chloride and sucrose density gradient centrifugation. Aichi virus (A846/88 strain) and poliovirus type 1 (PV-1, Sabin strain) were also grown and purified in the same manner. Purified viruses were examined under electron microscopy and by SDS-PAGE and were also used for the preparation of antisera and for ELISA. Immune sera were obtained from guinea pigs that were inoculated experimentally with Aichi virus (A846/88) and U-1 strain, respectively.

cDNA synthesis and cloning. The complete nucleotide sequence of purified U-1 strain was determined as described previously (Yamashita *et al.*, 1998), with modifications. Briefly, virion RNA was extracted using TRIzol (Invitrogen), following the instructions of the manufacturer. AMV reverse transcriptase (Promega) was used to create ss cDNA, with oligo$(dT)_{12–18}$ (Promega) and random six-residue primers (Takara). ds cDNA preparation and cloning into pBR322 and pUC19 were done as described previously (Supanaranond *et al.*, 1992; Takeda, 1989).

U-1 strain sequence-specific oligonucleotides were designed on the basis of sequences near the ends of the cloned cDNAs and were used for PCR. Oligo$(dT)_{33}$ was used for the extreme 3′ end of the genome. Six clones in a pGEM-T vector (Promega) background were obtained and sequenced to bridge the gaps between the pUC19 cDNA clones. The clones of the extreme 5′ end of the genome were obtained using the 5′ RACE kit (Roche), as described elsewhere (Sasaki *et al.*, 2001).

Stool and serum samples. Stool and serum samples from 2- to 4-year-old calves were obtained, together with pig serum samples, from slaughterhouses in the Aichi Prefecture. Human serum samples were obtained from the Japanese Red Cross. Horse serum samples were purchased from a market for laboratory use. Dog and cat serum samples were obtained from T. Kato, Kato Veterinary Hospital, Aichi Prefecture, Japan. Monkey serum samples were obtained from K. Asaoka, Kyoto University Primate Research Center, Kyoto, Japan. Calf stool samples were prepared as 10 % homogenates in PBS and centrifuged at 10 000 ***g*** for 20 min. Resultant supernatants were inoculated onto Vero cells and used for RT-PCR, as described below.

ELISAs. ELISAs was used to identify the reactivity of U-1 strain against Aichi virus and PV-1. ELISA plates (Nunc) were coated with 0·2 mg purified U-1 strain, Aichi virus or PV-1 per well and blocked with PBS/T (0·05 % Tween 20 and PBS) and 2 % BSA (Sigma). To each well of the plates were added a 100-fold or higher dilution of anti-U-1 strain or anti-Aichi virus serum. Plates were incubated overnight at 4 °C. After washing with PBS/T, peroxidase-labelled rabbit anti-guinea pig IgG (Zymed) in PBS/T with 1 % BSA was added to each well and incubated for 2 h at 37 °C. *o*-Phenylenediamine (Sigma) was used for colour development. After 30 min at room temperature, the reaction was stopped by addition of 4 M H_2SO_4. Absorbance readings were taken at 490 nm using a plate spectrophotometer (Corona Electric). Endpoint titres of the sera were defined as $A_{490} > 0·15$ (greater than three times the negative control well – without virus antigen).

RT-PCR. Primers for RT-PCR were designed based on the sequences of the Aichi virus and U-1 strain genomes. Oligonucleotide primer sequences were selected as follows: 10f (sense, 5′-GATGCTCCTCGGTGGTCTCA-3′; nt 7357) and 10r (anti-sense, 5′-GTCGGGGTCCATCACAGGGT-3′; nt 7987), which amplifies a 631 bp region of the 3D protein. RNA extraction from faecal samples was performed as described previously (Yamashita *et al.*, 2000). In brief, faecal extracts were centrifuged at 10 000 ***g*** for 20 min and the supernatant was collected for RT-PCR. As described by Jiang *et al.* (1992), 0·2 ml faecal extract was mixed with 0·1 ml 24 % polyethylene glycol-6000 and 1·5 M NaCl, stored at 4 °C overnight and centrifuged at 3000 ***g*** for 20 min. The pellet was suspended in 0·1 ml RNase-free water for RT-PCR. Virus RNA was extracted using TRIzol followed by isopropanol precipitation. Nucleic acid was suspended in reverse transcription mixture (Invitrogen) containing 1 μM oligo$(dT)_{15}$ (Promega) and 1 μM random primer (Takara) and incubated for 60 min at 37 °C. PCR mixtures containing primers were added directly to each of the reverse transcription mixtures and amplification was performed in a Thermal Cycler 9600 (Cetus, Perkin-Elmer) for 40 cycles (95 °C for 30 s, 55 °C for 30 s and 72 °C for 1 min). Analysis of the amplification product was performed by agarose minigel electrophoresis and confirmed as a distinct band by staining with ethidium bromide. Following RT-PCR, amplified products from positive faecal samples were purified by phenol/chloroform extraction. Purified RT-PCR products were then precipitated with ethanol and pelleted DNA was suspended in 10 mM Tris/HCl buffer (pH 8·5) and introduced into a pGEM-T vector (Promega). Aichi virus isolates (A846/89, A1156/87, M166/91 and P766/90) from patients with gastroenteritis (Yamashita *et al.*, 2000) were grown in Vero cells and used as samples for RT-PCR and DNA sequencing.

DNA sequencing and analyses. Inserts containing identified cDNA plasmid clones were used to determine nucleotide sequences using a SequiTherm LongRead Cycle Sequencing kit (Epicentre Technology) and an automated DNA sequencer (Model 4000, Li-Cor). The nucleotide sequence was analysed at least twice in both directions. The complete U-1 strain sequence has been submitted to the DDBJ/EMBL/GenBank databases under accession no. AB084788. The sequences amplified using the primers 10f and 10r have also been deposited in the databases under accession nos AB097152–AB097166.

Sequence comparisons between U-1 strain and Aichi virus (A846/88, accession no. AB010145 and AB040749) were made using the GCG sequence analysis package. A dendrogram was constructed using UPGMA (unweighted pair group method with averages) in the same package. The secondary structures of the 5′- and 3′-terminal nucleotides were predicted using the MFOLD program (Mathews *et al.*, 1999). The following nucleotide sequences were also obtained from DDBJ/EMBL/GenBank database: avian encephalomyelitis-like virus (AEV), AJ225173; bovine enterovirus type 1 (BEV-1), D00214; coxsackievirus A16 (CV-A16), U05876; coxsackievirus A21 (CV-A21), D00538; coxsackievirus B3 (CV-B3), M16572; encephalomyocarditis virus (EMCV), M81861; enterovirus type 70 (EV-70), D00820; equine rhinitis A virus (ERAV), L43052; equine rhinitis B virus (ERBV), X96871; foot-and-mouth disease virus type O (FMDV-O), X00871; hepatitis A virus (HAV), M14707; human parechovirus type 1 (HPeV-1), L02971; human rhinovirus type 2 (HRV-2), X02316;

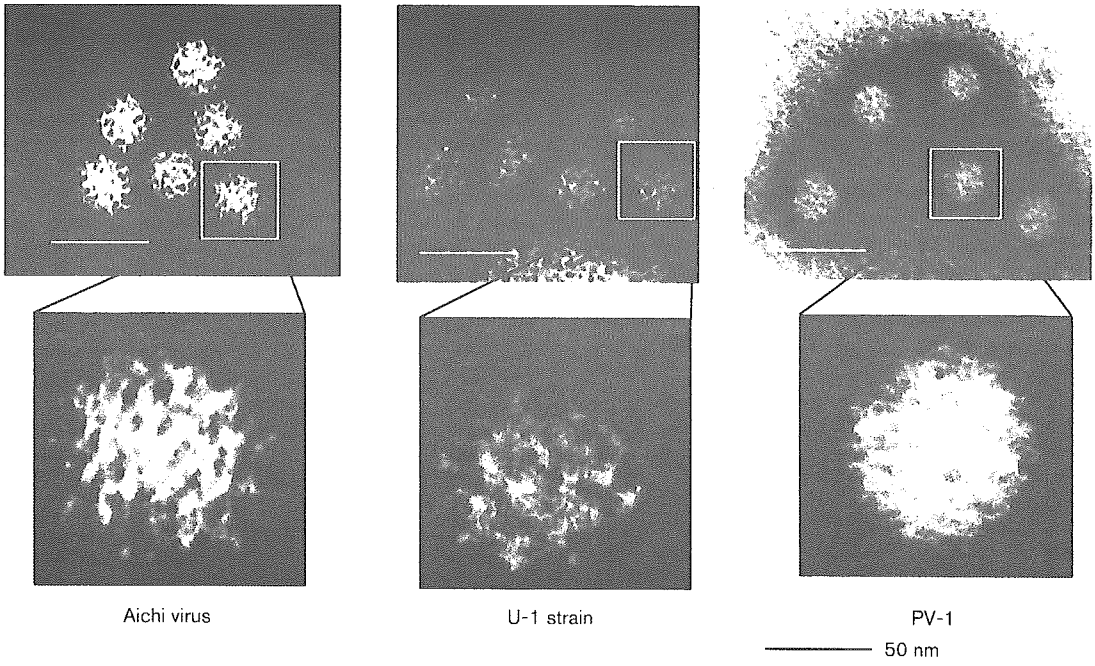

Fig. 1. Electron micrograph of Aichi virus, U-1 strain and PV-1 negatively stained with 2% phosphotungstic acid (pH 7·2) for 2 min.

human rhinovirus type 14 (HRV-14), K01087; Ljungan virus (LV), AF327920; poliovirus type 1 (PV-1), J02281; porcine enterovirus type 9 (PEV-9), Y14459; porcine teschovirus (PTV), AJ011380; and Theiler's murine encephalomyelitis virus (TMEV), M20301.

RESULTS

Biological properties of U-1 strain

U-1 strain was serially passaged in Vero cells, resulting in distinct CPE, and was titrated successfully using Vero cells grown in microplates (4×10^4–4×10^5 $TCID_{50}$ ml^{-1}). The addition of IUDR (10^{-4} M) to the Vero cell culture at the time of infection did not prevent CPE produced by U-1 strain. The results indicated that the nucleic acid type was RNA. It was stable to treatment with chloroform and acid (pH 3·5). Distinct virus particles could be seen following negative-staining for 2 min with phosphotungstic acid (pH 7·2) and had an average diameter of 30 nm, which resembles Aichi virus but is different from PV-1 (Fig. 1).

Reactivity of U-1 strain with EV, HPeV and Aichi virus

U-1 strain (4×10^3 $TCID_{50}$ ml^{-1}) could not be neutralized by 20 units of antibody against 64 prototype strains of EV, two prototype strains of HPeV and Aichi virus. U-1 strain antibody at a titre of 1 : 1000 could not neutralize these viruses. Purified PV-1, Aichi virus and U-1 strain (all at 0·2 mg ml^{-1}) were used to confirm reactivity with anti-Aichi virus and anti-U-1 strain sera using ELISA. Anti-U-1 strain at a titre of 1 : 16 000 did not react with Aichi virus and PV-1. However, anti-Aichi virus sera at a titre of 1 : 80 000 did react with U-1 strain at a titre of 1 : 40 000. In contrast, it did not react with PV-1 (Table 1). This result raised the possibility that there is a group-specific antigen between Aichi virus and U-1 strain.

Complete nucleotide sequence of U-1 strain

A total of 13 overlapping cDNA clones spanning the entire genome of U-1 strain were obtained and their nucleotide sequences determined. The RNA genome of U-1 strain consists of 8374 nt, excluding the poly(A) tail. A large ORF of 7392 nt, which encodes a potential polyprotein precursor of 2464 aa, was found, preceded at the 5′ end by 808 nt, and followed at the 3′ end by 174 nt and a poly(A) tail. The base composition was found to be 20·2% A, 21·7% G, 32·9% C and 25·2% U. The U-1 strain polyprotein sequence was analysed for potential cleavage

Table 1. Antigenic reactivity between U-1 strain and Aichi virus, as determined by ELISA

Virus	Reciprocal titre of antibody	
	U-1 strain	Aichi virus
U-1 strain	16 000	40 000
Aichi virus	< 100	80 000
PV-1	< 100	< 100

sites based on an alignment with the Aichi virus sequence. Possible cleavage sites for VP3/VP1, VP1/2A and 3B/3C were Q/A (Q/T in Aichi virus), Q/C (Q/G in Aichi virus) and Q/A (Q/G in Aichi virus), respectively. Other cleavage sites were found to be identical to those in Aichi virus (Table 2). In a previous study (Yamashita *et al.*, 1998), the first 32 nt of the Aichi virus genome were not identified and the published sequence (AB010145) exhibited 26 sequence differences from a sequence of Aichi virus (AB040749) published by Sasaki *et al.* (2001). These differences included ones that cause alterations in the 28 amino acid sequences in the VP0 region. The deduced amino acid sequence of the U-1 strain VP0 region was more analogous to that of the newly published ones than the earlier one. Therefore, U-1 strain RNA and polyprotein sequences were compared with the latter Aichi virus sequence (accession no. AB040749), as shown in Table 2.

Analysis of U-1 strain UTRs

The U-1 strain 5′ UTR was 64 bases longer than that of the Aichi virus 5′ UTR and its sequence identity was 49·3 % (Table 2). The predicted secondary structure of the 5′-terminal 116 nt consisted of three stem–loop domains. In particular, the first 50 bases, which comprised a stem–loop structure, was different by only one base from the Aichi virus stem–loop and the structure was found to be identical. The pyrimidine-rich tract was present between nt 765 and 771 and this was followed by the initiator methionine (AUG) at position 37. The 'spacer' of the U-1 strain was 26 nt longer than that of Aichi virus, which is 11 nt. An additional 89 nt were inserted before the pyrimidine-rich tract of U-1 strain (Fig. 2) in comparison with Aichi virus. The secondary structures of the internal ribosome entry site (IRES) are not known. The U-1 strain 3′ UTR was 63 bases shorter than that of the Aichi virus 3′ UTR but for the remainder the identity was 47·7 % (Table 2). The predicted secondary structure of the 3′ UTR of U-1 strain consisted of three ds hairpin stems but the secondary structure of the 3′ UTR of Aichi virus could not be defined (Yamashita *et al.*, 1998).

Analysis of U-1 strain coding regions

U-1 strain has a leader protein comprising 187 aa, which was 17 aa longer than Aichi virus, with an identity of 31 %. PAGE analysis of purified virus revealed three capsid proteins with molecular masses of 39, 29 and 22 kDa. Comparison of these proteins with those of Aichi virus and the molecular mass calculated for amino acids of the U-1 strain structural proteins indicated that the 39, 29 and 22 kDa polyproteins were VP0, VP1 and VP3, respectively (Fig. 3). The percentage identity between the U-1 strain and Aichi virus VP0 proteins (57·5 % amino acid identity) was similar to that comparing the VP2 sequences and higher than that of VP1 regions (33·2 %). VP1 sequences varied in length: 253 aa in Aichi virus and 267 aa in U-1 strain (Table 2).

The amino acid identity of the P2 and P3 proteins (non-structural proteins) varied from 44·9 % (3A) to 74·8 % (3D) between the U-1 strain and Aichi virus (Table 2). The amino acid sequence of the 2A region of U-1 strain had 57·4 % identity with Aichi virus and this conserved motif consisted of H-box/NC proteins and a transmembrane domain, as described elsewhere (Hughes & Stanway, 2000). Amino acid sequences of the 2C region were well aligned with Aichi virus, including the highly conserved motif GPPGTGKS, which is the nucleotide-binding domain of the putative picornavirus helicase. A catalytic triad formed by histidine, glutamate and cysteine in 3C was seen in U-1 strain at the same amino acid positions as in Aichi virus. The amino acid sequence of the 3D region aligned well,

Table 2. Comparisons of RNA and amino acids between U-1 strain and Aichi virus

	No. of RNA residues		Identity	No. of amino acids		Identity	Predicted N-terminal cleavage sites	
Regions	U-1 strain	Aichi virus	(%)	U-1 strain	Aichi virus	(%)	U-1 strain	Aichi virus
5′ UTR	808	744	49·3					
L	561	510	52·3	187	170	31·0		
VP0	1101	1110	61·9	367	370	57·5	Q/G	Q/G
VP3	669	669	63·7	223	223	58·3	Q/H	Q/H
VP1	801	759	53·3	267	253	33·2	Q/A	Q/T
2A	402	408	59·7	134	136	57·4	Q/C	Q/G
2B	495	495	58·5	165	165	51·5	Q/G	Q/G
2C	1005	1005	66·6	335	335	68·8	Q/G	Q/G
3A	282	285	56·7	94	95	44·9	Q/G	Q/G
3B	90	81	60·0	30	27	43·3	Q/A	Q/A
3C	576	570	58·3	192	190	47·9	Q/A	Q/G
3D	1410	1407	70·8	469	468	74·8	Q/S	Q/S
3′ UTR	174	237	47·7					
Total	8374	8280		2463	2432			

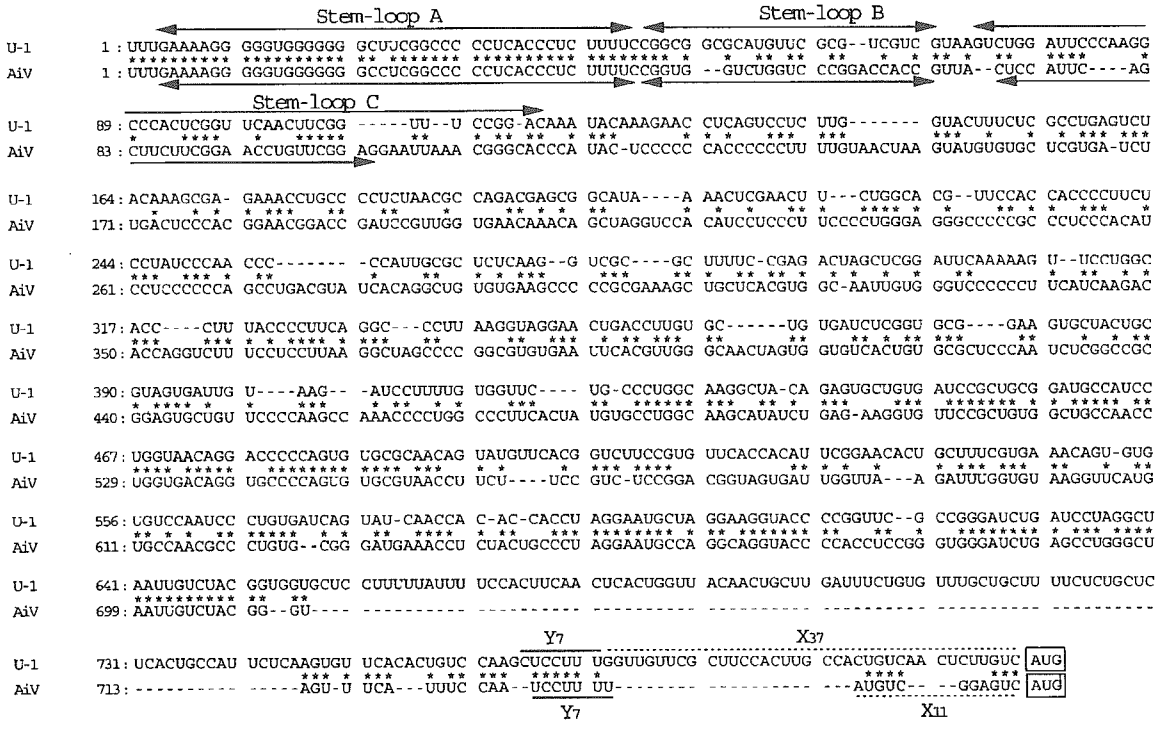

Fig. 2. Alignment of the 5′ UTRs of U-1 strain and Aichi virus. Arrows indicate the positions of three stem–loop structures. AiV, Aichi virus; Yn, pyrimidine-rich tract; Xm, nonconserved sequence; AUG, initiator methionine.

including the KDELR, YGDD and FLKR motifs of the RNA-dependent RNA polymerase.

Phylogenetic analysis

The relationships between the proteins of U-1 strain and those of other picornaviruses were examined by UPGMA, using amino acid multi-alignment of the P1, P2 and P3 regions. The dendrograms based on the P1, P2 and P3 proteins are depicted in Fig. 4. This confirmed that U-1 strain was clearly more closely related to Aichi virus than to any other picornaviruses, but the evolutionary distance

Table 3. Prevalence of U-1 strain and Aichi virus antibodies in different species

Species	No. tested	No. antibody positive	
		U-1 strain	Aichi virus
Human	190	0	129 (67·9%)
Monkey	242	0	0
Pig	139	0	0
Cattle	72	43 (59·7%)	0
Horse	5	0	0
Dog	22	0	0
Cat	9	0	0

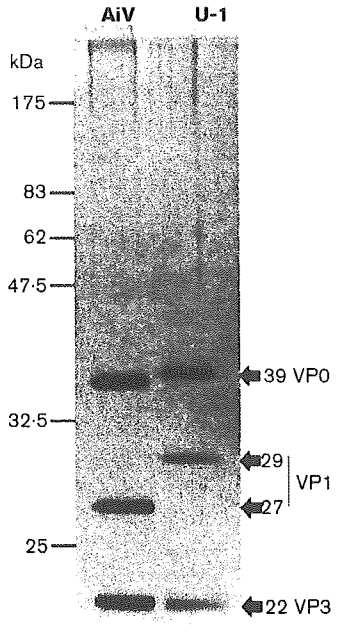

Fig. 3. SDS-PAGE of Aichi virus (AiV) and U-1 strain. The molecular masses (kDa) of VP0, VP1 and VP3 are shown on the right.

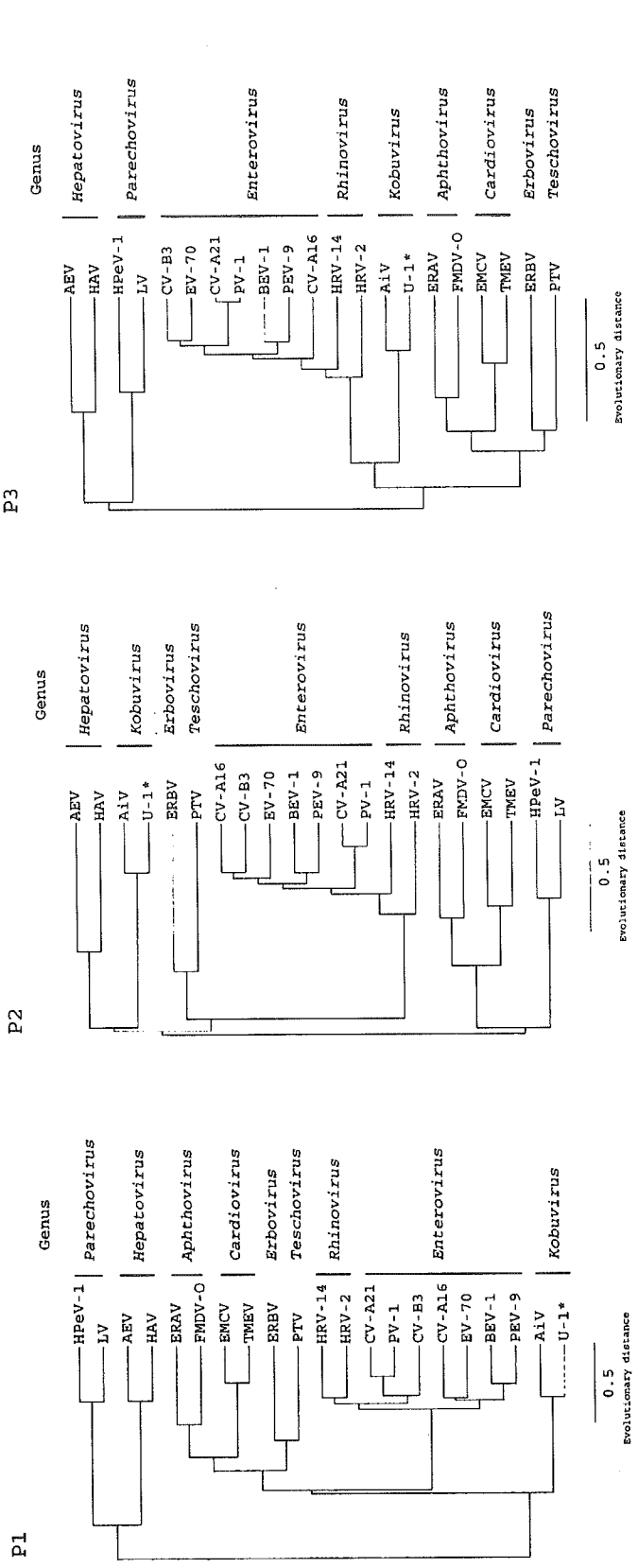

Fig. 4. Relationships between Aichi virus and other picornaviruses based on deduced amino acid differences of the P1, P2 and P3 proteins. The dendrograms were generated by evolutionary distances, as computed by UPGMA.

between U-1 strain and Aichi virus was equivalent to that between representative species in each genus of the *Picornaviridae*.

Prevalence of U-1 strain antibodies

Of 72 cattle sera, 43 (59·7 %) were positive for neutralizing antibody against U-1 strain at a titre of 1 : 16 or more. However, 190 human, 242 monkey, 139 pig, 5 horse, 22 dog and 9 cat sera did not neutralize U-1 strain at a serum dilution of 1 : 8. In contrast, antibody to Aichi virus was detected only in human samples (Table 3).

Detection of U-1 strain-like RNA in cattle stool samples

A total of 72 faecal specimens from healthy cattle were examined by passage in Vero cells and RT-PCR using the primer pair 10f and 10r. No U-1 strain-like virus could be isolated from the Vero cells but virus-specific RNA could be detected in 12 (16·7 %) of 72 samples using RT-PCR directly from faecal material. (The farms from which RNA-positive cattle were raised were located in different areas of the Aichi Prefecture.) The primer pair 10f and 10r could amplify products from U-1 strain and four Aichi virus isolates obtained from human faecal samples described elsewhere (Yamashita *et al.*, 1995). The sequences of the RNAs amplified from 12 cattle had approximately 90 % identity with U-1 strain and 60 % with the four Aichi virus isolates.

DISCUSSION

This study reports on the biological and serological properties and complete nucleotide sequence and genetic organization of a cytopathogenic agent, U-1 strain, isolated from HeLa cells on Vero cells. Biological and phylogenetic analyses revealed that this virus bears many similarities to Aichi virus and can be considered a new member of the genus *Kobuvirus*. Furthermore, epidemiological studies showing the prevalence of neutralizing antibody in some animals and the detection of RT-PCR products from cattle faeces warrants that this virus be a new species of the genus *Kobuvirus*, called bovine kobuvirus.

The identity of the 5′ UTRs between U-1 strain and Aichi virus was low. However, the first 50 bases differed by only one base and the secondary structure was identical. The 42 nt at the 5′ end of the genome formed a stable stem–loop structure and plays an essential role in the formation of virus particles as well as in RNA replication (Sasaki *et al.*, 2001). Our results strongly support this, suggesting the importance of this region and revealed that the secondary structure of this region is distinctive to this genus. The location of the pyrimidine tract and initiator methionine suggests that Aichi virus has an IRES that is most similar to type II IRES sequences (Yamashita *et al.*, 1998). When we compared the sequences of the 5′ UTRs of U-1 strain and Aichi virus, significant differences surrounding the pyrimidine tract could be identified. Therefore, the predicted secondary structure of the IRES consisting of stem–loop structures may be preserved in this genus, although the secondary structures of Aichi virus and U-1 strain IRES sequences are not known.

The major differences of Aichi virus from other picornaviruses are found in the coding region of the L protein, the absence of a VP0 cleavage site and a distinct form of the 2A protein (Stanway *et al.*, 2002). U-1 strain also exhibited this feature. When we compared amino acid identities between U-1 strain and Aichi virus, the percentage identity in the L protein (31·0 %) was lower than the 3A (44·9 %) or 3B (43·3 %) proteins. Aphthoviruses and cardioviruses also encode an L protein. The cleavage activity of the aphthovirus (FMDV) L protein has been well characterized (Piccone *et al.*, 1995; Strebel & Beck, 1986) and the cardiovirus TMEV L protein has been shown to be a zinc-binding protein that may play a role in restricting host cell growth (Chen *et al.*, 1995). The L proteins of U-1 strain and Aichi virus exhibited relatively low similarity to each other and no sequence identity to the L protein of aphthoviruses or cardioviruses. As a result, we cannot currently deduce a function for the L protein of U-1 strain and Aichi virus.

The Aichi virus VP0 protein has been shown to strongly react with convalescent-phase serum from patients (Yamashita *et al.*, 1991); therefore, it is probably exposed on the surface of the virions. However, the percentage identity of the VP1 protein between U-1 strain and Aichi virus was lower than that of the VP0 region. VP1 is the most exposed and immunodominant of the picornavirus capsid proteins (Rossmann *et al.*, 1985) and in enteroviruses, VP1 sequences correlate with neutralization type (Oberste *et al.*, 1999). Our results parallel this and suggest that the VP1 protein of kobuvirus was the most variable of the structural proteins.

The protein encoded at the 2A locus differs dramatically among picornaviruses and several distinct forms have been identified (Bazan & Fletterick, 1988; Donnelly *et al.*, 1997; Ryan & Drew, 1994; Yu & Lloyd, 1992). It has been reported that the 2A protein of Aichi virus as well as HPeV and AEV contain conserved motifs (H-box/NC) that are characteristic of a family of cellular proteins involved in the control of cell proliferation (Hughes & Stanway, 2000). The 2A protein of U-1 strain was similar to Aichi virus (57·4 % amino acid identity) and possessed these H-box/NC proteins. This percentage identity is higher than that of the 3C protein (47·9 %) and suggests that the 2A protein of kobuvirus may perform an important mechanism.

An atypical genome and codon base composition of Aichi virus has been pointed out elsewhere (Palmenberg & Sgro, 2002). The pyrimidine content (38 % C and 24 % U) of Aichi virus is higher than that of other picornaviruses. The triplet assignment in the standard genetic code is not random and the average picornavirus ratios for A : G : C : U are 30 : 31 : 19 : 20 (SD = 4). Aichi virus, however, has a

much higher than average C composition (C=28 %). In this study, 58 % of the U-1 strain base count was pyrimidine (33 % C and 25 % U). In the first codon base, the ratio of the U-1 strain base count was 23:32:25:21 for A:G:C:U. The high C composition in the genome is suspected to be a typical skew of kobuviruses.

The prevalence of the antibody to U-1 strain and positive results of RT-PCR in healthy cattle revealed that U-1 strain-like viruses are common between these domestic animals and are excreted in the faeces. Our HeLa cells were suspected to be infected with U-1 strain through a culture medium supplemented with calf serum, which had been possibly polluted with faeces. This discovery was not readily apparent, since it had grown in HeLa cells without any CPE. This was not necessarily surprising, because several species of picornaviruses have been identified as persistent infections *in vitro* (de la Torre *et al.*, 1985; Gibson & Righthand, 1985; Matteucci *et al.*, 1985; Roos *et al.*, 1982; Vallbracht *et al.*, 1984). Our results highlight the fact that HeLa cells kept in other laboratories may also have been contaminated with this virus.

BEV strains are well known to be endemic in cattle in many regions of the world. Although an infection of BEV is known to be asymptomatic, it can also been associated with diarrhoea and, on occasion, abortion (Ley *et al.*, 2002). The prevalence of anti-Aichi virus antibodies in man also suggests that there are likely to be many asymptomatic infections (Yamashita *et al.*, 1993, 1995). However, isolates of Aichi virus have been found only in patients with gastroenteritis. In this study, we detected kobuvirus-specific RNA in 12 (16·7 %) of 72 faecal samples from apparently healthy cattle by RT-PCR. These findings suggested that U-1 strain-like virus infections may be typically asymptomatic in cattle. However, we were not able to isolate a U-1 strain-like virus with Vero cells from the faeces of healthy cattle positive for kobuvirus-specific RNA. Like Aichi virus, this virus may be isolated only from symptomatic cattle. More epidemiological studies are required regarding the significance of this virus as a causative agent of some diseases of cattle. The development of RT-PCR for kobuvirus should prove useful for this study. To isolate U-1 strain-like viruses, another type of cell, such as bovine cells, may be required.

REFERENCES

Bazan, J. F. & Fletterick, R. J. (1988). Viral cysteine proteases are homologous to the trypsin-like family of serine proteases: structural and functional implications. *Proc Natl Acad Sci U S A* **85**, 7872–7876.

Chen, H. H., Kong, W. P. & Roos, R. P. (1995). The leader peptide of Theiler's murine encephalomyelitis virus is a zinc-binding protein. *J Virol* **69**, 8076–8078.

de la Torre, J. C., Davila, M., Sobrino, F., Ortin, J. & Domingo, E. (1985). Establishment of cell lines persistently infected with foot-and-mouth disease virus. *Virology* **145**, 24–35.

Donnelly, M. L., Gani, D., Flint, M., Monaghan, S. & Ryan, M. D. (1997). The cleavage activities of aphthovirus and cardiovirus 2A proteins. *J Gen Virol* **78**, 13–21.

Gibson, J. P. & Righthand, V. F. (1985). Persistence of echovirus 6 in cloned human cells. *J Virol* **54**, 219–223.

Hughes, P. J. & Stanway, G. (2000). The 2A proteins of three diverse picornaviruses are related to each other and to the H-rev 107 family of proteins involved in the control of cell proliferation. *J Gen Virol* **81**, 201–207.

Jiang, X., Wang, J., Graham, D. Y. & Estes, M. K. (1992). Detection of Norwalk virus in stool by polymerase chain reaction. *J Clin Microbiol* **30**, 2529–2534.

King, A. M. Q., Brown, F., Christian, P. & 8 other authors (2000). *Picornaviridae.* In *Virus Taxonomy. Seventh Report of the International Committee on Taxonomy of Viruses*, pp. 657–678. Edited by M. H. V. van Regenmortel, C. M. Fauquet, D. H. L. Bishop, E. B. Carstens, M. K. Estes, S. M. Lemon, J. Maniloff, M. A. Mayo, D. J. McGeoch, C. R. Pringle & R. B. Wickner. San Diego: Academic Press.

Ley, V., Higgins, J. & Fayer, R. (2002). Bovine enteroviruses as indicators of fecal contamination. *Appl Environ Microbiol* **68**, 3455–3461.

Marvil, P., Knowles, N. J., Mockett, A. P. A., Britton, P., Brown, T. D. K. & Cavanagh, D. (1999). Avian encephalomyelitis virus is a picornavirus and is most closely related to hepatitis A virus. *J Gen Virol* **80**, 653–662.

Mathews, D. H., Sabina, J., Zuker, M. & Turner, D. H. (1999). Expanded sequence dependence of thermodynamic parameters improves prediction of RNA secondary structure. *J Mol Biol* **288**, 911–940.

Matteucci, D., Paglianti, M., Giangregorio, A. M., Capobianchi, M. R., Dianzani, F. & Bendinelli, M. (1985). Group B coxsackieviruses readily establish persistent infections in human lymphoid cell lines. *J Virol* **56**, 651–654.

Niklasson, B., Kinnunen, L., Hornfeldt, B., Horling, J., Benemar, C., Hedlund, K. O., Matskova, L., Hyypia, T. & Winberg, G. (1999). A new picornavirus isolated from bank voles (*Clethrionomys glareolus*). *Virology* **255**, 86–93.

Oberste, M. S., Maher, K., Kilpatrick, D. R. & Pallansh, M. A. (1999). Molecular evolution of the human enteroviruses: correlation of serotyping with VP1 sequence and application to picornavirus classification. *J Virol* **73**, 1941–1948.

Palmenberg, A. C. & Sgro, J.-Y. (2002). Alignments and comparative profiles of picornavirus genera. In *Molecular Biology of Picornaviruses*, pp. 149–155. Edited by B. L. Semler & E. Wimmer. Washington, DC: ASM.

Piccone, M. E., Zellner, M., Kumosinski, T. F., Mason, P. W. & Grubman, M. J. (1995). Identification of the active-site residues of the L proteinase of foot-and-mouth disease virus. *J Virol* **69**, 4950–4956.

Pringle, C. R. (1999). Virus taxonomy at the XIth International Congress of Virology, Sydney, Australia, 1999. *Arch Virol* **144**, 2065–2070.

Roos, R. P., Richards, O. C., Green, J. & Ehrenfeld, E. (1982). Characterization of a cell culture persistently infected with the DA strain of Theiler's murine encephalomyelitis virus. *J Virol* **43**, 1118–1122.

Rossmann, M. G., Arnold, A., Erickson, J. W. & 9 other authors (1985). Structure of a human common cold virus and functional relationship to other picornaviruses. *Nature* **317**, 145–153.

Ryan, M. D. & Drew, J. (1994). Foot-and-mouth disease virus 2A oligopeptide mediated cleavage of an artificial polyprotein. *EMBO J* **13**, 928–933.

Sasaki, J., Kusuhara, Y., Maeno, Y., Kobayashi, N., Yamashita, T., Sakae, K., Takeda, N. & Taniguchi, K. (2001). Construction of an

infectious cDNA clone of Aichi virus (a new member of the family *Picornaviridae*) and mutational analysis of a stem–loop structure at 5′ end of the genome. *J Virol* **75**, 8021–8030.

Stanway, G., Hovi, T., Knowles, N. & Hyypia, T. (2002). Molecular and biological basis of picornavirus taxonomy. In *Molecular Biology of Picornaviruses*, pp. 17–24. Edited by B. L. Semler & E. Wimmer. Washington, DC: ASM.

Strebel, K. & Beck, E. (1986). A second protease of foot-and-mouth disease virus. *J Virol* **58**, 893–899.

Supanaranond, K., Takeda, N. & Yamazaki, S. (1992). The complete nucleotide sequence of a variant of coxsackievirus A24, an agent causing acute hemorrhagic conjunctivitis. *Virus Genes* **6**, 149–158.

Takeda, N. (1989). Complete nucleotide and amino acid sequences of enterovirus 70. In *Acute Hemorrhagic Conjunctivitis*, pp. 419–424. Edited by K. Ishii, Y. Uchida, K. Miyamura & S. Yamazaki. Tokyo: University of Tokyo Press.

Vallbracht, A., Hofmann, Wurster, L. K. G. & Flehmig, B. (1984). Persistent infection of human fibroblasts by hepatitis A virus. *J Gen Virol* **65**, 609–615.

Yamashita, T., Kobayashi, S., Sakae, K., Nakata, S., Chiba, S., Ishihara, Y. & Isomura, S. (1991). Isolation of cytopathic small round viruses with BS-C-1 cells from patients with gastroenteritis. *J Infect Dis* **164**, 954–957.

Yamashita, T., Sakae, K., Ishihara, Y., Isomura, S. & Utagawa, E. (1993). Prevalence of newly isolated, cytopathic small round virus (Aichi strain) in Japan. *J Clin Microbiol* **31**, 2938–2943.

Yamashita, T., Sakae, K., Kobayashi, S., Ishihara, Y., Miyake, T., Mubina, A. & Isomura, S. (1995). Isolation of cytopathic small round virus (Aichi virus) from Pakistani children and Japanese travelers from Southeast Asia. *Microbiol Immunol* **39**, 433–435.

Yamashita, T., Sakae, K., Tsuzuki, H., Suzuki, Y., Ishikawa, N., Takeda, N., Miyamura, T. & Yamazaki, S. (1998). Complete nucleotide sequence and genetic organization of Aichi virus, a distinct member of *Picornaviridae* associated with acute gastroenteritis in humans. *J Virol* **72**, 8408–8412.

Yamashita, T., Sugiyama, M., Tsuzuki, H., Sakae, K., Suzuki, Y. &, Miyazaki. Y. (2000). Application of a reverse transcription-PCR for identification and differentiation of Aichi virus, a new member of the picornavirus family associated with gastroenteritis in humans. *J Clin Microbiol* **38**, 2955–2961.

Yu, S. F. & Lloyd, R. E. (1992). Characterization of the roles of conserved cysteine and histidine residues in poliovirus 2A protease. *Virology* **186**, 725–735.

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Detection, Quantitation, and Phylogenetic Analysis of Noroviruses in Japanese Oysters

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Noroviruses (NVs) cause many cases of oyster- or clam-associated gastroenteritis in various countries. We collected 191 samples from Japanese oysters intended for raw consumption that had been harvested from the sea in two different areas between December 2001 and February 2002. To detect, quantitate, and phylogenetically analyze the NV genome in purified concentrates from the stomachs and digestive diverticula of these oysters, we amplified the NV capsid gene by reverse transcription-PCR. Phylogenetic analysis was performed by using the neighbor-joining method. We detected the NV genome in 17 of 191 oysters (9%). Phylogenetic analysis indicated genogroup I (Norwalk virus type) in 3 of the 17 oysters and genogroup II (Snow Mountain virus type) in the other 14. Both genogroups showed wide genetic diversity. To quantify the NV capsid gene in these oysters, we performed real-time PCR using genogroup-specific probes. More than 10^2 copies of the NV genome were detected in 11 of 17 oysters. The results suggested that about 10% of Japanese oysters intended for raw consumption harbored NVs, and more than 50% of those oysters in which NVs were detected had a large amount.

Noroviruses (NVs), which belong to the family *Caliciviridae*, cause acute gastroenteritis (25). According to the Japanese 2001 Food Poisoning Surveillance Report (http://www.mhlw.go.jp/topics/syokuchu), NVs accounted for 28% (7,358 of 25,862) of cases of food poisoning overall and 99% (7,358 of 7,371) of purely viral cases. In addition, NVs reportedly cause gastroenteritis in large numbers of patients in many countries, suggesting that NVs are distributed worldwide (21).

NVs, enteroviruses, astroviruses, and hepatitis virus type A are likely to be transmitted by shellfish such as oysters and clams (19). The Japanese, other Asians, and the French eat large amounts of raw fish or shellfish. Raw consumption causes many cases of food poisoning or infectious gastroenteritis as well as hepatitis (10). Previous epidemiologic studies have linked many cases of NV gastroenteritis to the oyster harvesting season (20). These viruses, known to persist in the environment, are found to be concentrated in shellfish (9).

No conventional cell culture method has been developed for the propagation of NVs (2). Detection of NVs has relied mainly on reverse transcription-PCRs (RT-PCR), enzyme-linked immunosorbent assays (ELISA), and electron microscopy (2). NVs can be divided into two distinct genogroups, I (Norwalk virus type) and II (Snow Mountain virus type) (25). Previous studies that used stool specimens from patients with nonbacterial gastroenteritis have demonstrated the presence of broad genetic diversity in each genogroup of NVs (5, 7, 23). However, the epidemiologic and phylogenetic characteristics of NVs in oysters remain obscure. In addition, to our knowledge, few investigations have quantified NVs in patients' stool samples or in shellfish. This information is important for the prevention of food poisoning caused by NVs and for determining the infective dose and index of contamination for NVs. We therefore carried out detection, quantitation, and phylogenetic analysis of NV genomes in Japanese oysters.

MATERIALS AND METHODS

Samples and preparation of viral suspension. A total of 191 Japanese oysters (*Crassosterea gigas* or *Crassosterea nippona*) were collected from fish distributors who were handling the harvest from two areas of the Setouchi sea (areas A and B) that were about 500 km apart. Both areas are located in the offshore region west of the Honshu island. The sample numbers and harvesting months for these areas are shown in Table 1. All oysters had been approved for raw consumption by meeting the provisions of the Regulation of Food Sanitation Law (i.e., <50,000 bacteria per g of oyster by standard plate count with <230 representing the coliform group). The fresh oysters were shucked, and the stomachs and digestive diverticula of the oysters were removed by dissection on the day of harvest and then weighed and homogenized in nine times their weight of phosphate-buffered saline without magnesium and calcium (8). In brief, the stomachs and digestive diverticula were placed in a solution of phosphate-buffered saline with the addition of 0.1 ml of antifoam B (Sigma, St. Louis, Mo.) The samples were homogenized for two 30-s intervals at a maximum speed of 18,000 rpm with an Omni-mixer (OCI Instruments, Waterbury, Conn.). Six milliliters of chloroform-butanol (1:1, vol/vol) was added to the homogenate. Then, the mixture was homogenized for an additional 30 s, and 170 ml of Cat-Floc T (Calgon, Elwood,

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TABLE 1. Oyster samples collected for this study

Collection mo and yr	No. of samples from:	
	Area A	Area B
December 2001	20	16
January 2002	40	20
February 2002	80	15
Total	140	51

Pa.) was added to the homogenate (8). In addition, to test the adequacy of RNA extraction, we added poliovirus type II (Sabin strain, corresponding to 10^2 copies of viral genes/assay) to the homogenate samples. After homogenate samples were centrifuged at 3,000 × *g* for 30 min at 4°C, all of the supernatant was layered onto 3 ml of 30% sucrose solution and ultracentrifuged at 154,000 × *g* for 3 h at 4°C. Then, the pellet was resuspended in 300 μl of doubly distilled water containing 20 U of RNase inhibitor (Promega, Madison, Wis.) and stored at −80°C until use.

RNA extraction, RT-PCR, sequencing, and real-time PCR. Viral RNA was extracted from 140 μl of viral suspension by using an available kit (QIAamp viral RNA mini kit; Qiagen GmbH, Hilden, Germany) and was finally suspended in 60 μl of doubly distilled water. Then, the RNA solution was treated with 5 U of DNase I (Takara, Tokyo, Japan). To amplify the partial capsid region of NVs by RT-PCR, we used genogroup-specific primers following reverse transcription as previously described (15). Primers for the first PCR were as follows: 5′-CGY TGG ATG CGN TTY CAT GA-3′ (COG1F; sense), 5′-CCA ACC CAR CCA TTR TAC A-3′ (G1-SKR; antisense), 5′-CAR GAR BCN ATG TTY AGR TGG ATG AG-3′ (COG2F; sense), and 5′-CCR CCN GCA TRH CCR TTR TAC AT-3′ (G2-SKR; antisense). Primers for the nested PCR were 5′-CTG CCC GAA TTY GTA AAT GA-3′ (G1-SKF; sense), 5′-CCA ACC CAR CCA TTR TAC A-3′ (G1-SKR; antisense), 5′-CNT GGG AGG GCG ATC GCA A-3′ (G2-SKF; sense), and 5′-CCR CCN GCA TRH CCR TTR TAC AT-3′ (G2-SKR; antisense) (12). We amplified the poliovirus type II VP1 gene by using the specific primers of 5′-AGC AAG CAC CGT ATT GAG CC-3′ (sense) and 5′-GTT TCA TGT CTG CTC CGT CTG-3′ (antisense) (unpublished data). The PCR protocol included incubation for 3 min at 94°C; then, 40 cycles of 94°C for 60 s, 50°C for 60 s, and 72°C for 2 min followed, with an additional 15 min for elongation at 72°C after the last cycle. This PCR procedure was repeated by using inner primers as a nested PCR (15). We used two types of positive controls and a viral gene-free negative control per five assays for amplification of NVs by PCR. Nested PCR is considered to be highly prone to false-positive results (2). Therefore, to check for conflicting PCR results, negative controls were included in each round of PCR. In addition, a first PCR round and a nested PCR round were performed independently. The size of the amplified DNA fragment was confirmed by electrophoresis on a 1.5% agarose gel. After purification of DNA fragments with a QIAquick PCR purification kit (Qiagen), the nucleotide sequence was determined with an automated DNA sequencer (ABI 310 DNA sequencer; Applied Biosystems, Foster City, Calif.) by using a dye terminator cycle sequencing ready reaction kit (Applied Biosystems) (15). We also quantified NV capsid genes by using real-time PCR as previously described (12). Fifteen microliters of DNase I-treated RNA solution was added to 15 μl of RT solution containing 100 mM Tris-HCl at pH 8.3, 150 mM KCl, 6 mM $MgCl_2$, 1 mM deoxynucleoside triphosphate mixture, 10 mM dithiothreitol, 75 pmol of random hexamer (Takara), 30 U of RNase inhibitor (Takara), and 200 U of reverse transcriptase [Superscript II, RNaseH(−); Invitrogen, San Diego, Calif.]. The RT mixture was incubated at 42°C for 60 min (RT reaction) and then at 70°C for 15 min (for the denaturation of reverse transcriptase). The real-time PCR mixture contained 5 μl of cDNA (RT product), 17.5 μl of TaqMan universal PCR master mix (Applied Biosystems), a 400 nM concentration of each primer, and fluorogenic probes [probes for genogroup I, 10.5 pmol of RING1(a)-TP and 3.5 pmol of RING1(b)-TP; probe for genogroup II, 3.5 pmol of RING2-TP] (12). The fluorogenic probes for real-time PCR were as follows: 5′-AGA TYG CGA TCY CCT GTC CA-3′ [RING1(a)-TP], 5′-AGA TCG CGG TCT CCT GTC CA-3′ [RING1(b)-TP], and 5′-TGG GAG GGC GAT CGC AAT CT-3′ (RING2-TP) (12). The following PCR protocol was used: 2 min at 50°C and 10 min at 95°C, 50 cycles of 95°C for 15 s, and 56°C for 60 s (12). Data were corrected by using an internal standard as previously described (12). In addition, we tested recovery of the NV genome by using highly purified NV particles obtained by a sucrose gradient method from patients with gastroenteritis.

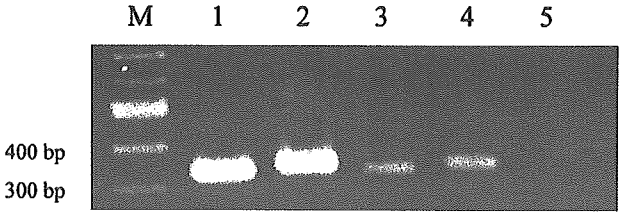

FIG. 1. Detection of NV capsid gene from oysters. Nested PCR products were electrophoresed on a 1.5% agarose gel. M, marker; lane 1, positive control for genogroup I (DSV395/90/Saudi Arabia, accession no. U04469); lane 2, positive control for genogroup II (Arg320, accession no. AF190817); lane 3, genogroup I amplicon from an oyster (A-9 strain in Fig. 2); lane 4, genogroup II amplicon from an oyster (B-3 strain in Fig. 2); lane 5, negative control (free of viral RNA). RT-PCR, nested PCR, and electrophoresis procedures are described in detail in Materials and Methods.

Phylogenetic analysis. Capsid sequences of reference strains of NVs were obtained from GenBank (13). These strains and accession numbers are shown in the legend for Fig. 2. Phylogenetic analysis was performed as previously described (13). Briefly, all NV capsid region sequences (between 238 and 253 bases) were aligned by using Genetix-Win version 5.0 software (Software Development, Tokyo, Japan). A phylogenetic tree was constructed by using the neighbor-joining technique, specifically, Kimura's two-parameter method. Reliability of the tree was estimated by using 1,000 bootstrap replications.

RESULTS

Detection and phylogenetic analysis of NVs in Japanese oysters. The NV capsid gene was amplified and detected in 17 of 191 oysters (9%). Amplicons from oysters were electrophoresed for comparison with corresponding NV protostrains (Fig. 1). All amplicons of NVs from oysters were sequenced successfully. As shown in Table 2, NV genomes were detected in 13 of 60 oysters in January 2002 (9 of 40 from area A and 4 of 20 from area B). NV genomes were detected in 4 of 95 oysters in February 2002 (3 of 80 from area A and 1 of 15 from

TABLE 2. Genogroups of and NV genome copy numbers in Japanese oysters

Collection mo and yr	Area	Strain	Genogroup	NV genome copy no.[a]	GenBank accession no.
January 2002	A	A-1	II	2.7×10^3	AB097905
	A	A-2	II	2.9×10^3	AB097906
	A	A-3	II	ND[b]	AB097907
	A	A-4	II	ND	AB097908
	A	A-5	II	7.5×10^3	AB097909
	A	A-6	II	1.6×10^2	AB097910
	A	A-7	I	2.9×10^4	AB097911
	A	A-8	II	ND	AB097912
	A	A-9	I	2.2×10^2	AB097913
	B	B-1	II	ND	AB097917
	B	B-2	II	6.2×10^2	AB097918
	B	B-3	II	1.5×10^2	AB097919
	B	B-4	I	2.4×10^2	AB097920
February 2002	A	A-10	II	ND	AB097914
	A	A-11	II	1.4×10^2	AB097915
	A	A-12	II	ND	AB097916
	B	B-5	II	4.8×10^2	AB097921

[a] Units are copy numbers of NV genome per oyster.
[b] ND, not detected ($<10^2$ copies/oyster).

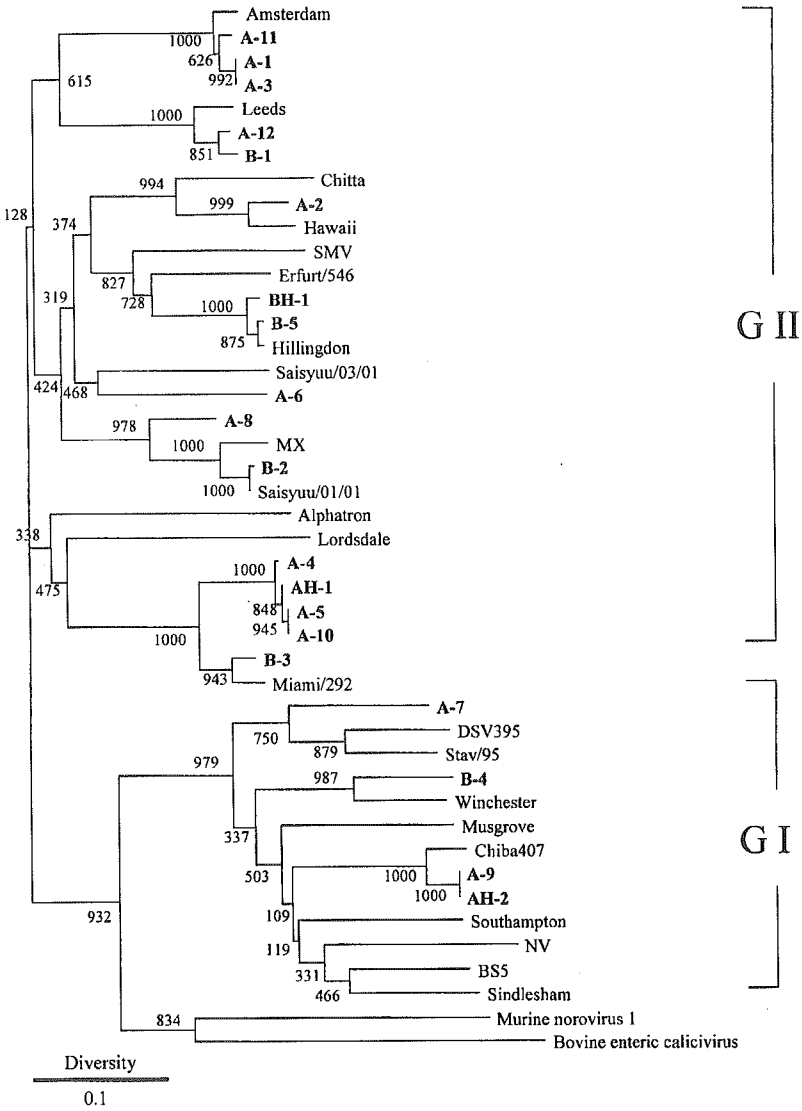

FIG. 2. Phylogenetic tree construct based on partial sequences of the capsid gene of NVs. The distance was calculated by Kimura's two-parameter method, and the tree was plotted by using the neighbor-joining method. Numbers at each branch indicate bootstrap values for the clusters supported by that branch. Accession numbers of our strains are indicated. A and B refer to the areas of harvest, and positive samples from this study are designated in boldface type. AH and BH refer to human NV strains detected in patients with gastroenteritis living near sites A and B. GI, genogroup I; GII, genogroup II. Strains and GenBank accession numbers were as follows: DSV395/90/Saudi Arabia, U04469; NLV/Stav/95/Nor, AF145709; Hu/NLV/Winchester/94/UK, AJ277609; Hu/NLV/Sindlesham/95/UK, AJ277615; BS5/Germany, AF093797; NV/8FiiA/68/US, M87661; Hu/NLV/Musgrove/89/UK, AJ277614; Hu/NLV/Chiba407/1987/JP, AB042808; Southampton/91/UK, L07418; Hu/NLV/Leeds/90/UK, AJ277608; Hu/NLV/Amsterdam/98-18/1998/NET, AF195848; NLV/Miami/292/1994/US, AF414410; HV/NLV/Hillingdon/90/UK, AJ277607; NLV/Erfurt/546/00/DE, AF427118; SMV/76/US (Snow Mountain virus), U70059; Hu/NLV/GII/Hawaii virus/1971/US, U07611; Aichi 124-89, AB031013; NV/Saisyuu/03/01, AB091399; NV/Saisyuu/01/01, AB091398; MX/89/Mexico, U22498; Hu/NLV/Alphatron/98-2/1998/NET, AF195847; Lordsdale/93/UK, X86557; AH-1, AB111894; BH-1, AB111896; BH-2, AB111895; murine norovirus 1, AY228235; bovine enteric calicivirus, AJ11099.

area B). No NV genome was detected in oysters harvested in December 2001. A phylogenetic tree constructed by the neighbor-joining method is shown in Fig. 2. Of 17 amplicons, 3 were classified into genogroup I (Norwalk virus type), while 14 were classified into genogroup II (Snow Mountain virus type). These strains could be subdivided into five clusters of genogroup II and two clusters of genogroup I. Both genogroups were detected in areas A and B. Several amplicons harvested in area A (A-4, A-5, and A-10; A-1, A-3, and A-11) were closely related genetically (i.e., exhibiting less than 1% genetic diversity). Interestingly, the sequences of amplicons B-2 Saisyuu/01/01 and (derived from a Korean oyster) were closely related genetically, although we only partially sequenced the capsid gene of these strains. The two areas of harvest were about 500 km apart, but no regionally distinctive distribution of an NV genogroup was evident in our phylogenetic tree. Nucleotide diver-