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ORIGINAL ARTICLE

Novel monoclonal antibodies broadly reactive to human recombinant sapovirus-like particles

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ABSTRACT

Sapovirus (SaV), a member of the family *Caliciviridae*, is an important cause of acute epidemic gastroenteritis in humans. Human SaV is genetically and antigenically diverse and can be classified into four genogroups (GI, GII, GIV, and GV) and 16 genotypes (7 GI [GI.1–7], 7 GII, [GII.1–7], 1 GIV and 1 GV), based on capsid sequence similarities. Monoclonal antibodies (MAbs) are powerful tools for examining viruses and proteins. PAI myeloma cells were fused with spleen cells from mice immunized with a single type of recombinant human SaV virus-like particles (VLPs) (GI.1, GI.5, GI.6, GII.3, GIV, or GV). Sixty-five hybrid clones producing MAbs were obtained. Twenty-four MAbs were characterized by ELISA, according to their cross-reactivity to each VLP (GI.1, GI.5, GI.6, GII.2, GII.3, GII.4, GII.7, GIV, and GV). The MAbs were classified by this method into: (i) MAbs broadly cross-reactive to all GI, GII, GIV and GV strains; (ii) those reactive in a genogroup-specific; and (iii) those reactive in a genotype-specific manner. Further analysis of three broadly cross-reactive MAbs with a competitive ELISA demonstrated that at least two different common epitopes are located on the capsid protein of human SaVs in the four genogroups. The MAbs generated and characterized in this study will be useful tools for further study of the antigenic and structural topography of the human SaV virion and for developing new diagnostic assays for human SaV.

Key words cross-reactivity, monoclonal antibody, sapovirus.

Sapovirus, a member of the family *Caliciviridae*, causes gastroenteritis in humans and is a significant public health problem (1–5). SaV was originally identified by EM of fecal specimens obtained during a gastroenteritis outbreak (6, 7).

The SaV capsid is composed of 90 dimers of capsid protein (VP1) (8). SaV has a ~7.5 kb genome of single-stranded positive-sense RNA that is predicted to encode two or three ORFs. The functions of proteins encoded by ORF2 and ORF3 are unknown. However, ORF1 encodes nonstructural proteins and VP1 (9, 10). VP1 is likely produced by cleavage of the ORF1 polyprotein by viral protease or by translation from subgenomic RNA (3′-coterminal with the virus genome), or both (11, 12). A tripeptide, MEG, conserved among human SaV strains, is probably the putative VP1 start on the subgenomic RNA.

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List of Abbreviations: CBB, Coomassie brilliant blue; DAB, diaminobenzidine; EM, electron microscopy; G, genogroup; HRPO, horseradish peroxidase; MAb, monoclonal antibody; NoV, norovirus; OD, optical density; OPD, ortho-phenylenediamine; ORF, open reading frame; P domain, protrusion domain; TPBS, Tween 20 phosphate-buffered saline; S domain, shell domain; SaV, sapovirus; *Sf9*, *Spodoptera frugiperda*; VLP, virus-like particles; VP1, capsid protein; WB, western blotting.

VP1 expressed from the putative subgenomic RNA or putative VP1-encoding construct in insect or mammalian cells self-assembles into virus-like particles that are morphologically similar to native SaV (12, 13–20). SaV VP1 has an apparent molecular mass of 60 kDa (11, 12, 21). Based on their complete VP1 sequences, SaVs are classified into at least five genogroups: GI, GII, GIII, GIV and GV. GI, GII, GIV and GV infect humans, and GIII infects porcine species (9). Human SaVs can be further separated into 16 genetic clusters (seven GI [GI.1–7], seven GII, [GII.1–7], one GIV and one GV) (22).

Because there is no cell-culture system or small-animal model for human SaV, SaV VLPs have been used as models of SaV virion for immunogenic, antigenic and structural studies. The capsid proteins of human SaVs have high antigenic diversity (16, 17, 20, 21, 23). However, little information is available about whether specific regions of the VP1 are important for antigenic specificity, and whether type-specific and/or cross-reactive epitopes are present in SaVs.

Monoclonal antibodies are powerful tools for the study of viruses and proteins. A panel of MAbs against SaV VLPs would be valuable for antigenic and structural analysis as well as useful for developing new diagnostic assays for human SaV. In this study, we established such a panel of MAbs broadly cross-reactive to all human SaV genogroups, GI, GII, GIV and GV, as well as MAbs specific to either genogroups or genotypes.

MATERIALS AND METHODS

Generation of recombinant baculoviruses

DNA fragments corresponding to the putative subgenomic RNA region of the genome (approximately 2.3 kb in length) of GI.6 Nichinan (GenBank accession number AB455803 [24]), GII.3 20082029 (AB630068 [22]), GII.3 D1711 (AB522391 [25]), GII.3 Kushiro5 (AB455793 [26]), GII.3 Nayoro4 (AB455794 [26]), GII.4 Kumamoto6 (AB429084 [1, 22]), GII.7 20072248 (AB630067 [22]), and GIV Yakumo8 (AB455795 [26]) were amplified by PCR with KOD-Plus-DNA polymerase (Toyobo) as previously described (27). A forward primer (5′-CAGATCTGCA GCGGCCGCATGGAGGN$_{8-10}$ [N indicates strain specific sequence]-3′) included a NotI site (underlined), and a common reverse primer (5′-GTCCCAGGAAAGGATCC TTTTTTTTTTTTTTTTTTTTTTTTTTTTTT-3′) included a BamHI site (underlined). The amplified fragments were cloned into NotI- and BamHI-digested baculovirus transfer vector pVL1392 (Orbigen, San Diego, CA, USA) with an In-Fusion Advantage PCR Cloning Kit (Takara, Shiga, Japan), according to the manufacturer's protocol. *Escherichia coli* HST08 premium competent cells (Takara) were used to transform and propagate the transfer plasmid. Sequencing analysis confirmed the consensus sequence of each strain. An insect cell line derived from *Sf9* (Riken Cell Bank, Tsukuba, Japan) was co-transfected with a linearized wild-type *Autographa californica* nuclear polyhedrosis virus DNA (Baculo-Gold, BD Bioscience, Franklin Lakes, NJ, USA) and the transfer vectors carrying the human SaV putative subgenomic RNA region by the lipofectin-mediated method as specified by the manufacturer (Gibco BRL, Gaithersburg, MD, USA).

Expression and purification of human sapovirus virus-like particles

For larger scale expression of the SaV capsid proteins, BTl-Tn-5B1–4 (Tn5), an insect cell line derived from *Trichoplusia ni* (Invitrogen, San Diego, CA, USA), was infected with recombinant baculoviruses at a multiplicity of infection of 10 and incubated for 7 days at 26°C, as previously described (28). Eight novel VLPs derived from GI.6 Nichinan, GII.3 20082029, GII.3 D1711, GII.3 Kushiro5, GII.3 Nayoro4, GII.4 Kumamoto6, GII.7 20072248 and GIV Yakumo8 were purified as follows. Intact cells, cell debris, and progeny baculoviruses were removed by centrifugation at 10,000 *g* for 60 min. The supernatant was then centrifuged at 174,899 *g* for 3 hr in a Beckman SW32-Ti rotor rotor (Beckman Coulter, Fullerton, CA, USA). The resulting pellet was resuspended in EX-CELL 405 Serum Free medium (SAFC Biosciences, Lenexa, KS, USA) at 4°C overnight and the debris removed by centrifugation at 10,000 *g* for 30 min at 4°C. The supernatant was then centrifuged at 154,000 *g* for 2 hr in a Beckman TLA55. The resulting pellet was resuspended in EX-CELL 405 Serum Free medium (SAFC Biosciences) at 4°C overnight, and the debris removed by centrifugation at 10,000 *g* for 5 min at 4°C. After mixing with 2.1 g of CsCl in MilliQ water, the sample was centrifuged at 148,862 *g* for 24 hr at 10°C in a Beckman SW55-Ti rotor. After fractionation (20 × 250 μL each), each aliquot was diluted with EX-CELL 405 medium, and centrifuged at 154,000 *g* for 2 hr at 4°C in a Beckman Coulter TLA55 rotor. The resulting pellet was resuspended in EX-CELL 405 medium. Seven VLPs derived from GI.1 Mc114 (AY237422 [27]), GI.5 Yokote1 (AB253740 [17]), GII.2 Mc10 (AY237420 [10]), GII.3 C12 (AY603425 [10]), GII.3 Syd53 (DQ104360 [29]), GIV Syd3 (DQ104357 [29]) and GV NK24 (AY646856 [30]) were expressed and purified as previously described (14, 16, 17, 20).

Preparation of monoclonal antibodies

The PAI myeloma cell line (kindly provided by M. Kotani, Tokyo Metropolitan Institute of Medical Science,

Tokyo, Japan) was cultured in Dulbecco's modified Eagle's medium with 15% FCS. The MAbs were prepared essentially as previously described (31, 32), with minor modifications. Briefly, BALB/c mice were primed intraperitoneally with 1–10 μg of purified VLPs (GI.1 Mc114, GI.5 Yokote1, GI.6 Nichinan, GII.3 Syd53, GIV Syd3, or GV NK24 VLPs) per mouse, in the presence of adjuvant. The mice received booster inoculations four times at 1 week intervals, the final injection of antigen being administered i.v. Three to four days after the final injection, the animals were killed and cells from their spleens fused with the myeloma cells. The culture medium of the hybridomas that resulted from successful fusions was screened for reactivity by ELISA. ELISA plates were coated with VLPs as described below. Positive hybridomas were cloned by limiting dilution and antibody-producing clones were grown and stored in liquid nitrogen until used for further tests. Finally, ascites fluid was prepared by injecting the hybridomas into pristane-primed mice and used to provide the MAbs for this study. The isotype and subclass of each MAb were determined by ELISA with anti-mouse subtype MAbs (Cappel Laboratories, West Chester, PA, USA) or an IC kit (IsoQuick, Sigma, Saint Louis, MO, USA).

All animal procedures conformed to the Animal Handling and Ethical Regulations of the University of Hyogo and the provisions of the Declaration of Helsinki. This research project was approved by the Ethics Committee of the University of Hyogo.

Enzyme-linked immunosorbent assay

An indirect ELISA, with slight modifications, was used to screen and characterize the MAbs (31). Briefly, 96-well microplates (Nunc-immune plate, Nunc, Roskilde, Denmark) were coated with 100 ng of VLPs/well in 50 μL of PBS (pH 7.2) overnight at 4°C. The plates were washed with TPBS and blocked with 5% skim milk in PBS for 1 hr at 37°C. The MAbs (ascites, appropriate dilution, 50–100 μL) were added and incubated for 1 hr at 37°C. After washing with TPBS, 50 μL of a 1:2000 dilution of HRPO–conjugated goat anti-mouse immunoglobulin G (IgG), IgM, or IgA (Cappel Laboratories) was added to each well and incubated for 1 hr at 37°C. After washing, 50 μL of OPD-H_2O_2 (0.5 mg of ortho-phenylenediamine/mL, 0.002% H_2O_2, 0.1 M citrate-phosphate buffer, pH 5.5) was added, incubated for 10–20 min, and the optical densities measured at 490 and 655 nm with a Microplate Reader (Model 550, Bio-Rad, Richmond, CA, USA).

To further characterize the epitopes recognized by these MAbs, a competitive indirect ELISA was performed as previously described (33, 34) with a slight modification. Briefly, VLP was used to coat 96-well microplates overnight at 4°C at a concentration of 50–100 ng/well in PBS (pH 7.2). In separate tubes, MAbs at a concentration of 5–500 ng/mL (depending on the VLPs used for the coating) were added to decreasing concentrations of competitor VLP (10, 1, 0.1 and 0.01 μg/mL) in PBS (pH 7.2) containing 1% skim milk, and then incubated overnight at 4°C. As a control, MAb without competitor VLP was included in each plate. The VLP-coated plates were washed and blocked with 5% skim milk for 1 hr at 37°C, 100 μL of each of the VLP-MAb reaction mixtures was added to duplicate wells, and the plates were incubated for 2 hr at 37°C. The reactivity of the antibody to the competitors was expressed as B/B_0, where B is the amount of antibody bound to the coating antigen in the presence of the competitor, and B_0 is the amount in the absence of the competitor.

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis

The VLPs (0.5–1 μg per track) were suspended in electrophoresis sample buffer containing 1% SDS, 10% 2-mercaptoethanol, 50 mM Tris-HCl (pH 6.8), 0.0025% phenol red, and 10% glycerol. The samples were boiled for 2 min, then subjected to 10% SDS-PAGE (catalog no. EMP-8020; 1 mm thick, 8 cm long, 7 cm wide; Iwaki, Tokyo, Japan) at a constant current of 20 mA for 1.5–2 hr. The gels were stained with 0.1% CBB (Sigma) or silver staining kit (Ez stain Silver, Atto, Tokyo Japan).

Immunoblotting

Western blotting analysis was performed as previously described (31, 35) with slight modifications. Briefly, after electrophoresis, the gel was transferred electrophoretically to a nitrocellulose membrane (0.45 μm pore size, Millipore, Bedford, MA, USA) in a semidry transfer (EPM-8460; Iwaki) at a constant current of 70 mA for 2–3 hr. The strips were prepared and incubated overnight at room temperature with the MAbs (ascites fluid) at a dilution of 1:500–1000. The blots were incubated with a 1:2000 dilution of HRPO-conjugated goat anti-mouse IgG, IgM, and IgA (Bio-Rad) for 1 hr at 37°C. The strips were soaked in a solution of DAB (0.5 mg/mL, 3–'diaminobenzidine, 0.001% H_2O_2, 50 mM Tris-HCl buffer, pH 6.0) to detect the antigen-antibody complexes on the strips.

Sequence analysis

To confirm the sequences of the panel of plasmids used in this study, nucleotide sequence analysis was performed with a Big Dye Terminator (version 3.1) Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Tokyo, Japan) and an automated sequencer, the 3130 Genetic Analyzer (Applied Biosystems). Nucleotide sequences were

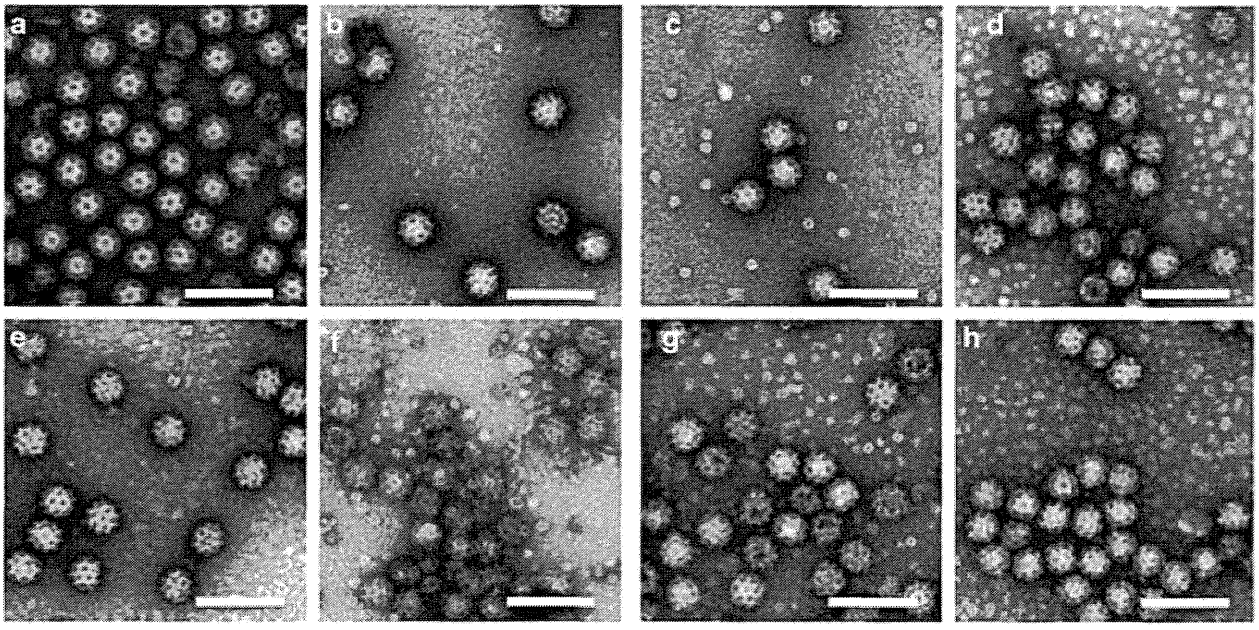

Fig. 1. Electron micrographs of novel SaV VLPs. VLPs derived from (a) SaV GI.6 Nichinan, (b) GII.3 20082029, (c) GII.3 D1711, (d) GII.3 Kushiro5, (e) GII.3 Nayoro4, (f) GII.4 Kumamoto6, (g) GII.7 20072248 and (h) GIV Yakumo8 have diameters of 41–43 nm. Purified VLPs were stained with 4% uranyl acetate (pH 4) and examined by an electron microscope (TEM-1400; JOEL, Japan) operating at 80 kV. Scale bars = 100 nm.

assembled with the program Sequencher, version 4.10.1 (Gene Codes, Ann Arbor, MI, USA). Nucleotide and amino acid sequences were analyzed with GENETYX Mac software, version 16.0.4 (Genetyx, Tokyo, Japan).

RESULTS

Expression of novel human sapovirus virus-like particles

Sapovirus capsid proteins were expressed in an insect cell, Tn5, and VLPs were purified by CsCl equilibrium density gradient centrifugation as described in Materials and Methods. EM analysis of eight purified SaV VLPs from GI.6 Nichinan, GII.3 20082029, GII.3 D1711, GII.3 Kushiro5, GII.3 Nayoro4, GII.4 Kumamoto6, GII.7 20072248 and GIV Yakumo8 showed cuplike surface depressions and almost homologous particles with diameters of approximately 41–43 nm (Fig. 1). The morphology of these recombinant particles is very similar to those we have observed in previous studies (14, 16, 19, 20).

Isolation of sapovirus monoclonal antibodies

BALB/c mice were immunized intraperitoneally with purified SaV VLPs and their spleen cells fused with PAI myeloma cells. Sixty-five hybrid clones producing MAbs were obtained from six different recombinant human SaV VLPs from GI.1 Mc114, GI.5 Yokote1, GI.6 Nichinan, GII.3 Syd53, GIV Syd3 or GV NK24. Twenty-four MAbs were obtained from mouse ascites and classified into five groups, according to their patterns of ELISA reactivity with 15 VLPs (GI.1 [$n = 1$], GI.5 [$n = 1$], GI.6 [$n = 1$], GII.2 [$n = 1$], GII.3 [$n = 6$], GII.4 [$n = 1$], GII.7 [$n = 1$], GIV [$n = 2$], and GV [$n = 1$]) (Table 1). The MAbs were grouped as follows: MAbs cross-reacting with all GI, GII, GIV and GV (group A, $n = 8$); GI-specific or type-specific MAbs (group B, $n = 7$); GII-specific or type-specific MAbs (group C, $n = 3$); GIV-specific MAbs (group D, $n = 2$); and GV-specific MAbs (group E, $n = 4$).

Monoclonal antibodies cross-reactive with heterologous genogroups and genotypes.

Eight group A MAbs showed binding to all GI, GII, GIV and GV VLPs examined in this study, although their reactivity to each VLP was different. Four MAbs (namely, 5C9, 1A1, 5C1 and 6C4) reacted consistently and strongly with VLPs from different genogroups and genotypes when the OD ratios between samples and PBS were greater than 10. On the other hand, another four MAbs (8127, 6D3, 3D2 and 4G7) reacted either strongly or moderately (OD ratio: 5–9) with GI, GIV and/or GV VLPs, and weakly (OD ratio 1–4) with GII VLPs in ELISA (Table 1). Among these eight MAbs, three (5C9, 1A1 and 8127) were further confirmed

Table 1. Reactivities of monoclonal antibodies with 15 SaV VLPs in ELISA

				Reactivity[a]														
				GI[b]			GII									GIV		GV
Group	MAb	Immunogen	Isotype	1[c] Mc114	5 Yokote1	6 Nichinan	2 Mc10	3 C12	3 20082029	3 D1711	3 Syd53	3 Kushiro5	3 Nayoro4	4 Kumamoto6	7 20072248	1 Syd3	1 Yakumo8	1 NK24
A	5C9	Nichinan	IgG1	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
	1A1	Nichinan	IgG1	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
	5C1	Nichinan	IgG1	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
	6C4	Nichinan	IgM	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
	8127	Yokote1	IgG1	+++	+++	+++	+	+	+	+	+	++	+	+	+	++	++	+++
	6D3	Nichinan	IgG1	++	+++	+++	++	+	+	+	NT	++	++	++	++	++	++	++
	3D2	Nichinan	IgG1	+	++	+++	++	+	+	++	NT	+	++	+	+	+	+	++
	4G7	Nichinan	IgG1	+	NT	+++	+	+	+	+	NT	+	+	+	+	+	+	+
B	616	Yokote1	IgG1	++	+++	++	–	–	–	–	–	–	–	–	–	–	–	–
	1325	Mc114	IgG1	++	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	5001	Mc114	IgG1	++	–	NT	–	–	NT	NT	–	NT	NT	NT	NT	–	NT	–
	4357	Mc114	IgG1	++	–	NT	–	–	NT	NT	–	NT	NT	NT	NT	–	NT	–
	627	Yokote1	IgG1	–	++	NT	–	–	NT	NT	–	NT	NT	NT	NT	–	NT	–
	7F8	Nichinan	IgM	–	–	++	–	–	–	–	NT	–	–	–	–	–	–	–
	1F2	Nichinan	IgM	–	–	++	–	–	–	–	NT	–	–	–	–	–	–	–
C	1803	Syd53	IgG3	–	–	–	++	++	++	++	++	++	++	++	++	–	–	–
	8083	Syd53	IgG3	–	–	NT	++	++	NT	NT	++	NT	NT	NT	NT	–	–	–
	1015	Syd53	IgG2b	–	–	–	–	++	++	++	++	++	+	–	–	–	–	–
D	819	Syd3	IgG1	–	–	NT	–	–	NT	NT	–	NT	NT	NT	NT	++	++	–
	806	Syd3	IgG1	–	–	NT	–	–	NT	NT	–	NT	NT	NT	NT	++	++	–
E	1496	NK24	IgG1	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+++
	155	NK24	IgG1	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+++
	4971	NK24	IgG1	–	–	NT	–	–	NT	NT	–	NT	NT	NT	NT	–	NT	+++
	1052	NK24	IgG1	–	–	NT	–	–	NT	NT	–	NT	NT	NT	NT	–	NT	+++
	anti-SaV serum			+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
	PBS			–	–	–	–	–	–	–	–	–	–	–	–	–	–	–

[a]Reactivities: +++, strong (OD ratio of sample/PBS > 10); ++, moderate (5–9); +, weak (1–4); –, negative (< 1); NT, not tested.
[b]Genogroup
[c]Genotype (22)

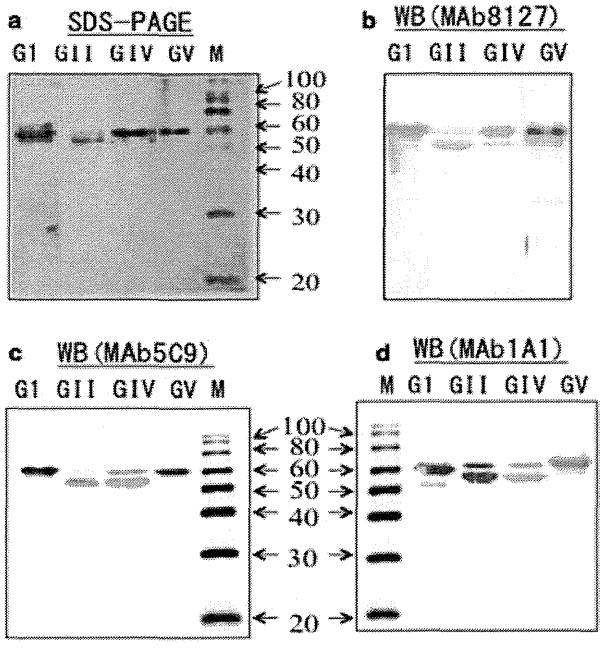

Fig. 2. SDS-PAGE of purified SaV VLPs and western blots. VLPs from (a, b) GI (GI.5 Yokote1) and (c, d) GI.6 Nichinan, GII (20072248), GIV (Yakumo8) or GV (NK24) were separated by SDS-PAGE. The proteins were stained with (a) CBB or transferred to a nitrocellulose sheet which was then incubated with (b) MAbs 8127, (c) 5C9 or (d)1A1. Lane M, molecular mass markers (kDa).

by WB analysis to react with GI, GII, GIV and GV SaV VP1 with an apparent molecular mass of about 60 kDa (Fig. 2). These results indicate that SaV capsid protein carries at least one epitope common to GI, GII, GIV and GV SaV. Protein bands smaller than 60 kDa are likely truncated VP1 as previously described, though direct evidence is lacking (14).

Genogroup- or genotype-specific monoclonal antibodies

Seven group B MAbs reacted exclusively with GI VLPs, but not with any other VLPs from GII, GIV and GV SaVs. MAb 616 bound to three GI SaV VLPs (GI.1 Mc114, GI.5 Yokote1 and GI.6 Nichinan) in a genogroup-specific manner, whereas MAbs 1325, 5001, 4357, 627, 7F8 and 1F2 bound to VLPs in a genotype-specific manner (Table 1). Three group C MAbs (1803, 8083 and 1015) showed reactivity only with GII VLPs. MAb 1803 reacted to all GII VLPs (GII.2, -3, -4, and -7) examined, but not to GI, GIV and GV VLPs. On the other hand, MAb 1015 reacted with all GII.3 VLPs, but not with GII.2, GII.4, GII.7, GI, GIV and GV VLPs, demonstrating that this MAb is likely to be GII.3-specific (Table 1). Two group D MAbs (819 and 806) were specific to GIV (Syd3 and Yakumo8) VLPs and did not bind to GI, GII and GV VLPs. Four group E MAbs (1496, 155, 4971 and 1052) were specific to GV VLPs (Table 1).

Epitopes recognized by cross-reactive monoclonal antibodies

Three MAbs classified as group A (5C9, 1A1 and 8127) had broad reactivity to VLPs derived from all human SaVs genogroups (GI, GII, GIV and GV). The specificity of these MAbs was further examined by competitive ELISA. When GI.6 Nichinan VLP was used as the coating antigen, binding of MAb 5C9 was completely inhibited. In addition, binding of MAb 5C9 was similarly blocked by three heterotypic VLPs (GII.7 20072248, GIV Yakumo8 and GV NK24) (Fig. 3a). Similar results were obtained when GII.7 20072248 VLP was used as the coating antigen. Binding of MAb 5C9 was completely inhibited by homotypic GII.7 VLP and also by three heterotypic VLPs, GI.6 Nichinan, GIV Yakumo8 and GV NK24 (Fig. 3b). The results were similar when two other VLPs, GIV Yakumo8 and GV NK24, were used as the coating antigens (data not shown). Binding of MAb 1A1 to four VLPs (GI.6 Nichinan, GII.7 20072248, GIV Yakumo8 and GV NK24), as well as inhibition by homotypic and heterotypic VLPs was exactly the same as that of MAb 5C9 (Fig. 3c, d), suggesting these two MAbs (5C9 and 1A1) recognize a common epitope. On the other hand, binding of MAb 8127 to GI.5 Yokote1 and GV NK24 VLPs was different. Binding of MAb 8127 to GI VLP was strongly blocked by both homotypic GI VLP and heterotypic GV VLP (Fig. 3e) and binding to GV VLP was similarly inhibited by both homotypic GV VLP and heterotypic GI VLP (Fig. 3f). However, inhibition by two other competitor VLPs, GII.7 20072248 and GIV Yakumo8, was incomplete (Fig. 3e, f), demonstrating that the epitope recognized by MAb 8127 is different from that of MAbs 5C9 and 1A1.

DISCUSSION

In this study, we established 65 hybridoma cell lines from six mice immunized with six SaV VLPs, and characterized 24 MAbs in detail. These MAbs were classified into group A (MAbs broadly cross-reactive to all GI, GII, GIV and GV strains), and groups B–E (genogroup-specific or genotype-specific MAbs). We also obtained another 27 MAbs specific to particular strains: five to GI.1 Mc114, three to GI.5 Yokote1, five to GII.3 Syd3, five to GIV Syd3, and nine to GV NK24 (data not shown). In addition, 14 MAbs were positive by ELISA but negative by WB. We did not further characterize these 41 MAbs in this study.

Group A MAbs are broadly reactive to all GI, GII, GIV and GV SaV VLPs by both ELISA and WB (Table 1

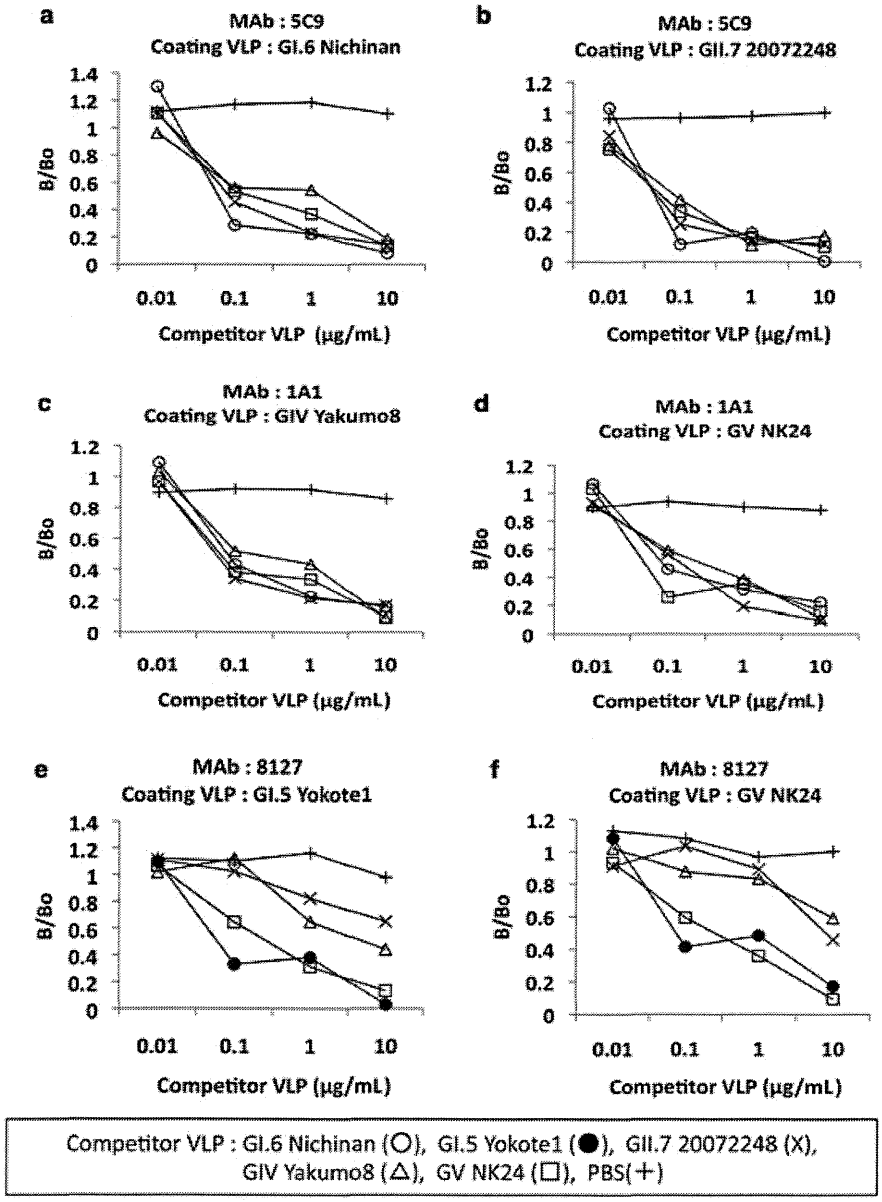

Fig. 3. Competitive ELISA to differentiate epitope recognition. Microplates were coated with VLPs from (a) GI.6 Nichinan, (b) GII.7 20072248, (c) GIV Yakumo8, (e) GI.5 Yokote1, or (d, f) GV NK24. A reaction mixture of the various concentrations of the competitor VLPs and a MAb was added to the plates and binding of the MAb to the coated VLPs was measured as described in Materials and Methods. The competitor VLPs used in this experiments were ○, GI.6 Nichinan; ●, GI.5 Yokote1; X, GII.7 20072248; △, GIV Yakumo8; □, GV NK24. PBS(+) was used as a control without competitor. (a, b) MAbs 5C9, (c, d) 1A1 and (e, f) 8127 were incubated with the competitor VLPs.

and Fig. 2), indicating that cross-reactive epitope(s) is/are present on the SaV VP1 in these four genogroups. Based on the competition ELISA, the epitopes recognized by MAbs 5C9 and 1A1 seems to be common to them but different to that of MAb 8127 (Fig. 3). In ELISA, the reactivity of three MAbs (5C9, 1A1 and 5C1) was consistent and strong to GI, GII, GIV and GV VLPs. MAbs 8127 reacted strongly with GI and GV VLPs but weakly with GII and GIV VLPs (Table 1). The different reactivities between different genogroups of SaV VP1 partly supports the possibility of distinct epitopes for MAbs 5C9, 1A1 and MAb 8127.

The X-ray crystallographic structure of human SaV has not been reported, but cryo-electron microscopy has revealed structural similarities between human SaV VLPs and NoV VLPs (8). X-ray crystallographic studies of

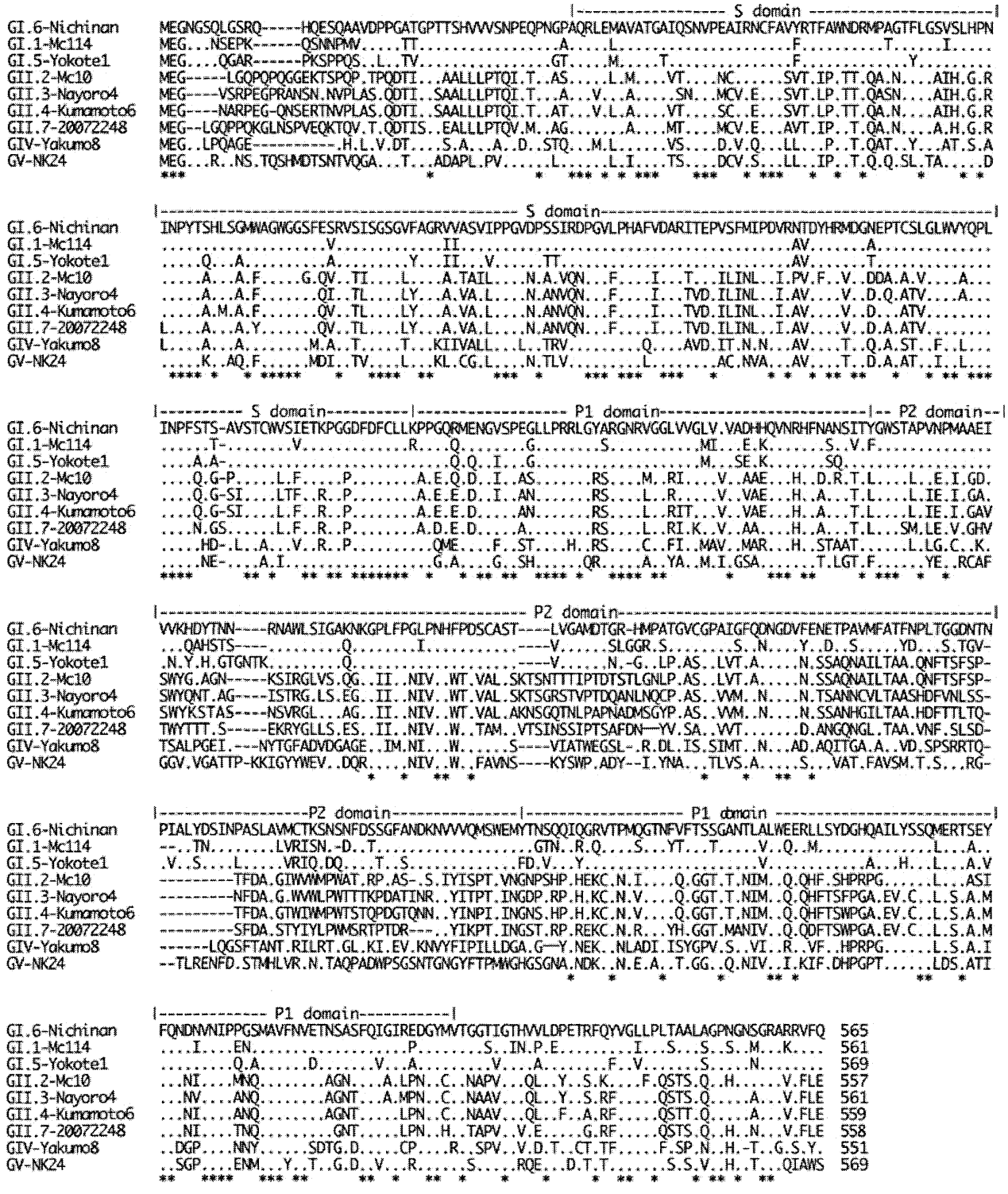

Fig. 4. Amino acid alignment of nine representative SaV VP1 protein sequences. Predicted amino acid sequences of GI.6 Nichinan, GI.1 Mc114, GI.5 Yokote1, GII.2 Mc10, G II.3 Nayoro4, GII.4 Kumamoto6, GII.7 20072248, GIV Yakumo8 and GV NK24 VP1 are shown. Asterisks indicate conserved amino acids among the 15 SaV strains used in this study. Dots indicate identical amino acid residues. The extents of S and P domains indicated in the above sequences were inferred from published information about NoV VP1 (8).

human NoV VLPs has revealed that NoV VP1 has the following two principal domains: a shell (S) domain, and a protrusion (P) domain that is further divided into three subdomains called N-terminal P1, P2 and C-terminal P1 (36). The NoV S domain and P1 subdomains are highly and moderately conserved, respectively. The P2 domain is highly variable among NoV strains (8, 37), this domain is likely to be the key determinant of strain specificity and antigenicity (37). MAbs broadly cross-reactive with NoV VLPs have been described (31, 34, 38–41), these studies demonstrating that the epitopes are located in the S domain (40, 41) or C-terminal P1 domain (38, 42) in the NoV VP1.

Hyper-immune sera raised against human SaV VLPs has revealed distinct antigenicity among different human SaV genogroups and genotypes (15–17, 20, 21, 23, 43). Genogroup- and genotype-specific MAbs (group B–E MAbs) were also isolated in this study. These MAbs will be useful tools for further study of the antigenic determinant of human SaV. Amino acid sequence homology of VP1 among the 15 SaV strains is 28.1% (data not shown). Despite these significant amino acid sequence variations among different genogroups and genotypes of SaV VP1, the predicted S domain is relatively more conserved than the P2 domains (Fig. 4). From the amino acid sequence alignment, the antigenic determinant is likely to be present in the predicted P2 domain in human SaV, and common epitopes may occur in the predicted S or P1 domains, although further experiments are necessary.

In conclusion, we have established a panel of MAbs that are reactive with human SaV VLPs in a broad, genogroup-specific or genotype-specific manner. The broadly reactive MAbs are of particular interest as possible reagents for the development of human SaV detection or diagnostic assays (i.e., ELISA or immunochromatography) in clinical settings, because ELISA using hyperimmune sera shows narrow reactivity to specific genogroups (15–17, 21, 23, 43). Because human SaVs have also been detected in clams (25,44), oysters (45) and environmental water (46–51), broadly reactive MAbs may also become valuable tools for concentrating or removing human SaVs from food or environmental specimens.

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DISCLOSURE

The authors declare no financial or commercial conflicts of interest.

REFERENCES

1. Harada S., Okada M., Yahiro S., Nishimura K., Matsuo S., Miyasaka J., Nakashima R., Shimada Y., Ueno T., Ikezawa S., Shinozaki K., Katayama K., Wakita T., Takeda N., Oka T. (2009) Surveillance of pathogens in outpatients with gastroenteritis and characterization of sapovirus strains between 2002 and 2007 in Kumamoto Prefecture, Japan. *J Med Virol* **81:** 1117–27.
2. Iturriza-Gomara M., Elliot A.J., Dockery C., Fleming D.M., Gray J.J. (2009) Structured surveillance of infectious intestinal disease in pre-school children in the community: 'The Nappy Study'. *Epidemiol Inf* **137:** 922–31.
3. Monica B., Ramani S., Banerjee I., Primrose B., Iturriza-Gomara M., Gallimore C.J., Brown D.W., Fathima M., Moses P.D., Gray J.J., Kang G. (2007) Human caliciviruses in symptomatic and asymptomatic infections in children in Vellore, South India. *J Med Virol* **79:** 544–51.
4. Pang X.L., Lee B.E., Tyrrell G.J., Preiksaitis J.K. (2009) Epidemiology and genotype analysis of sapovirus associated with gastroenteritis outbreaks in Alberta, Canada: 2004–2007. *J Infect Dis* **199:** 547–51.
5. Svraka S., Vennema H., van der Veer B., Hedlud K-O., Thorhagen M., Siebenga J., Duizer E., Koopmans M. (2010) Epidemiology and genotype analysis of emerging sapovirus-associated infections across Europe. *J Cln Microbiol* **48:** 2191–8.
6. Chiba S., Sakuma Y., Kogasaka R., Akihara M., Horino K., Nakao T., Fukui S.J. (1979) An outbreak of gastroenteritis associated with calicivirus in an infant home. *J Med Virol* **4:** 249–54.
7. Chiba S., Sakuma Y., Kogasaka R., Akihara M., Terashima H., Horino K., Nakao T. (1980) Fecal shedding of virus in relation to the days of illness in infantile gastroenteritis due to calicivirus. *J Inf Dis* **142:** 247–9.
8. Chen R., Neill J.D., Noel J.S., Hutson A.M., Glass R.I., Estes M.K. (2004) Inter- and intragenus structural variations in caliciviruses and their functional implications. *J Virol* **78:** 6469–79.
9. Farkas T., Zhong W.M., Jing Y., Huang P.W., Espinosa S.M., Martinez N., Morrow A.L., Ruiz-Palacios G.M., Pickering L.K., Jiang X. (2004) Genetic diversity among sapoviruses. *Arch Virol* **149:** 1309–23.
10. Katayama K., Miyoshi T., Uchino K., Oka T., Tanaka T., Takeda N., Hansman G.S. (2004) Novel recombinant sapovirus. *Emerg Infect Dis* **10:** 1874–6.
11. Hansman G.S., Oka T., Takeda N. (2008) Sapovirus-like particles derived from polyprotein. *Virus Res* **137:** 261–5.
12. Oka T., Yamamoto M., Miyashita K., Ogawa S., Katayama, K., Wakita T., and Takeda N. (2009) Self-assembly of sapovirus recombinant virus-like particles from polyprotein in mammalian cells. *Microbiol Immunol* **53:** 49–52.
13. Guo M., Qian Y., Chang K.O., Saif L.J. (2001) Expression and self-assembly in baculovirus of porcine enteric calicivirus capsids

into viruslike particles and their use in an enzyme-linked immunosorbent assay for antibody detection in swine. *J Clin Microbiol* **39:** 1487–93.
14. Hansman G.S., Natori K., Oka T., Ogawa S., Tanaka K., Nagata N., Ushijima H., Takeda N., Katayama K. (2005) Cross-reactivity among sapovirus recombinant capsid proteins. *Arch Virol* **150:** 21–36.
15. Hansman G.S., Natori K., Ushijima H., Katayama K., Takeda N. (2005) Characterization of polyclonal antibodies raised against sapovirus genogroup five virus-like particles. *Arch Virol* **150:** 1433–7.
16. Hansman G.S., Oka T., Sakon N., Takeda N. (2007) Antigenic diversity of human sapoviruses. *Emerg Infect Dis* **13:** 1519–25.
17. Hansman G.S., Saito H., Shibata C., Ishizuka S., Oseto M., Oka T., Takeda N. (2007) Outbreak of gastroenteritis due to sapovirus. *J Clin Microbiol* **45:** 1347–9.
18. Numata K., Hardy M.E., Nakata S., Chiba S., Estes M.K. (1997) Molecular characterization of morphologically typical human calicivirus Sapporo. *Arch Virol* **142:** 1537–52.
19. Oka T., Hansman G.S., Katayama K., Ogawa S., Nagata N., Miyamura T., Takeda N. (2006) Expression of sapovirus virus-like particles in mammalian cells. *Arch Virol* **151:** 399–404.
20. Oka T., Miyashita K., Katayama K., Wakita T., Takeda N. (2009) Distinct genotype and antigenicity among genogroup II sapoviruses. *Microbiol Immunol* **53:** 417–20.
21. Farkas T., Deng X., Ruiz-Palacios G., Morrow A., Jiang X. (2006) Development of an enzyme immunoassay for detection of sapovirus-specific antibodies and its application in a study of seroprevalence in children. *J Clin Microbiol* **44:** 3674–9.
22. Oka T., Mori K., Iritani N., Harada S., Ueki Y., Iizuka S., Mise K., Murakami K., Wakita T., Katayama K. (2012) Human sapovirus classification based on complete capsid nucleotide sequences. *Arch Virol* **157:** 349–52.
23. Jiang X., Cubitt D.W., Berke T., Zhong W.N., Dai X., Nakata S., Pickering L.K., Matson D.O. (1997) Sapporo-like human caliciviruses are genetically and antigenetically diverse. *Arch Virol* **142:** 1813–27.
24. Iwakiri A., Ganmyo H., Yamamoto S., Otao K., Mikasa M., Kizoe S., Katayama K., Wakita T., Takeda N., Oka T. (2009) Quantitative analysis of fecal sapovirus shedding: identification of nucleotide substitutions in the capsid protein during prolonged excretion. *Arch Virol* **154:** 689–93.
25. Iizuka S., Oka T., Tabara K., Omura T., Katayama K., Takeda N., Noda M. (2010) Detection of sapoviruses and noroviruses in an outbreak of gastroenteritis linked genetically to shellfish. *J Med Virol* **82:** 1247–54.
26. Ishida S., Yoshizumi S., Miyoshi M., Ikeda T., Okui T., Katayama K., Takeda N., Oka T. (2008) Characterization of sapoviruses detected in Hokkaido, Japan. *Jpn J Infect Dis* **61:** 504–6.
27. Hansman G.S., Katayama K., Maneekarn N., Peerakome S., Khamrin P, Yonusin S., Okitsu S., Nishio O., Takeda N., Ushijima H. (2004) Genetic diversity of norovirus and sapovirus in hospitalized infants with sporadic cases of acute gastroenteritis in Chiang Mai, Thailand. *J Clin Microbiol* **42:** 1305–07.
28. Li T.C., Takeda N., Miyamura T., Matsuura Y., Wang J.C., Engvall H., Hammar L., Xing L., Cheng R.H. (2005) Essential elements of the capsid protein for self-assembly into empty virus-like particles of hepatitis E virus. *J Virol* **79:** 12999–3006.
29. Hansman G.S., Takeda N., Katayama K., Tu E.T., McIver C.J., Rawlinson W.D. (2006) Genetic diversity of sapovirus in children, Australia. *Emerg Infect Dis* **12:** 141–3.
30. Guntapong R., Hansman G.S., Oka T., Ogawa S., Kageyama T., Pongsuwanna Y. (2004) Norovirus and sapovirus infections in Thailand. *Jpn J Infect Dis* **57:** 276–8.
31. Kitamoto N., Tanaka T., Natori K., Takeda N., Nakata S., Jiang X., Estes M.K. (2002) Cross-reactivity among several recombinant calicivirus virus-like particles (VLPs) with monoclonal antibodies obtained from mice immunized orally with one Type of VLP. *J Clin Microbiol* **40:** 2459–65.
32. Kohler G., Milstein C. (1975) Continuous cultures of fused cells secreting antibody of predefined specificity. *Nature* **256:** 495–7.
33. Hale A.D., Tanaka T., Kitamoto N., Ciarlet M., Jiang X., Takeda N., Brown D.W.G., Estes M.K. (2000) Identification of an epitope common to genogroup 1 Norwalk-like viruses. *J Clin Microbiol* **38:** 1656–60.
34. Hardy M.E., Tanaka T., Kitamoto N., White L.J., Ball J.M., Jiang X., Estes M.K. (1996) Antigenic mapping of the recombinant Norwalk virus capsid protein using monoclonal antibodies. *Virology* **217:** 252–62.
35. Towbin H., Staehelin T., Gordon J. (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc Natl Acad Sci USA* **76:** 4350–4.
36. Prasad B.V., Hardy M.E., Dokland T., Bella J., Rossmann M.G., Estes M.K. (1999) X-ray crystallographic structure of the Norwalk virus capsid. *Science* **286:** 287–90.
37. Hansman G.S., Natori K., Shirato-Horikoshi H., Ogawa S., Oka T., Katayama K., Tanaka T., Miyoshi T., Sakae K., Kobayashi S., Shinohara M., Uchida K., Nakurai S., Shinozaki K., Okada M., Seto Y., Kamata K., Nagata N., Tanaka K., Miyamura T., Takeda N. (2006) Genetic and antigenic diversity among noroviruses. *J Gen Virol* **87:** 909–19.
38. Shiota T., Okame M., Takahasi S., Khamrin P., Takagi M., Satou K., Masuoka Y., Yagyu F., Shimizu Y., Kohno H., Mizuguchi M., Okitsu S., Ushijima H. (2007) Characterization of a broadly reactive monoclonal antibody against norovirus genogroups I and II: recognition of a novel conformational epitope. *J Virol* **81:** 12298–306.
39. Tanaka T., Kitamoto N., Jiang X., Estes M.K. (2006) High efficiency cross-reactive monoclonal antibody production oral immunization with recombinant Norwalk virus-like particles. *Microbiol Immunol* **50:** 883–8.
40. Yoda T., Terano Y., Suzuki Y., Yamazaki K., Oishi I., Kuzuguchi T., Kawamoto H., Utagawa E., Takino K., Oda H., Shibata T. (2001) Characterization of Norwalk virus GI specific monoclonal antibodies generated against *Escherichia coli* expressed capsid protein and the reactivity of two broadly reactive monoclonal antibodies generated against GII capsid towards GI recombinant fragments. *BMC Microbiol* **1:** 24.
41. Yoda T., Suzuki Y., Terano Y., Yamazaki K., Sakon N., Kuzuguchi T., Oda H., Tsukamoto T. (2003) Precise characterization of norovirus (Norwalk-like virus)-specific monoclonal antibodies with broad reactivity. *J Clin Microbiol* **41:** 2367–71.
42. Parker T.D., Kitamoto N., Tanaka T., Hutson A.M., and Estes M.K. (2005) Identification of genogroup I and genogroup II broadly reactive epitopes on the norovirus capsid. *J Virol* **79:** 7402–9.
43. Hansman G.S, Guntapong R., Pongsuwanna Y., Natori K., Katayama K., Takeda N. (2006) Development of an antigen ELISA to detect sapovirus in clinical stool specimens. *Arch Virol* **151:** 551–61.
44. Hansman G.S., Oka T., Okamoto R., Nishida T., Toda S., Noda M., Sano D., Ueki Y., Imai T., Omura T., Nishio O., Kimura H., Takeda

N. (2007) Human sapovirus in clams, Japan. *Emerg Infect Dis* **13:** 620–2.
45. Ueki Y., Shoji M., Okimura Y., Miyota Y., Masago Y., Oka T., Katayama K., Takeda N., Noda M., Miura T., Sano D., Omura T. (2010) Detection of Sapovirus in oysters. *Microbiol Immunol* **54:** 483–6.
46. Hansman G.S., Sano D., Ueki Y., Imai T., Oka T., Katayama K., Takeda N., Omura T. (2007) Sapovirus in water, Japan. *Emerg Infect Dis* **13:** 133–5.
47. Haramoto E., Katayama K., Phanuwan C., Ohgaki S. (2008) Quantitative detection of sapoviruses in wastewater and river water in Japan. *Lett Appl Microbiol* **46:** 408–13.
48. Iwai M., Hasegawa S., Obara M., Nakamura K., Horimoto E., Takizawa T., Kurata T., Sogen S., Shiraki K. (2009) Continuous presence of noroviruses and sapoviruses in raw sewage reflects infections among inhabitants of Toyama, Japan (2006 to 2008). *Appl Environ Microbiol* **75:** 1264–70.
49. Kitajima M., Oka T., Haramoto E., Katayama H., Takeda N., Katayama K., Ohgaki S. (2010) Detection and genetic analysis of human sapoviruses in river water in Japan. *Appl Environ Microbiol* **76:** 2461–7.
50. Kitajima M., Haramoto E., Phanuwan C., Katayama H. (2011) Genotype distribution of human sapoviruses in wastewater in Japan. *Appl Environ Microbiol* **77:** 4226–9.
51. Sano D., Preze-Stautu U., Guix S., Pinto R.S., Miura T., Okabe S., Bosch A. (2011) Quantification and genotyping of human sapoviruses in the Llobregat River catchment, Spain. *Appl Environ Microbiol* **77:** 1111–4.

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Whole-genome analysis of two bovine rotavirus C strains: Shintoku and Toyama

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Rotavirus C (RVC) has been detected frequently in epidemic cases and/or outbreaks of diarrhoea in humans and animals worldwide. Because it is difficult to cultivate RVCs serially in cell culture, the sequence data available for RVCs are limited, despite their potential economical and epidemiological impact. Although whole-genome sequences of one porcine RVC and seven human RVC strains have been analysed, this has not yet been done for a bovine RVC strain. In the present study, we first determined the nucleotide sequences for five as-yet underresearched genes, including the NSP4 gene, from a cultivable bovine RVC, the Shintoku strain, identified in Hokkaido Prefecture, Japan, in 1991. In addition, we elucidated the ORF sequences of all segments from another bovine RVC, the Toyama strain, detected in Toyama Prefecture, Japan, in 2010, in order to investigate genetic divergence among bovine RVCs. Comparison of segmental nucleotide and deduced amino acid sequences among RVCs indicates high identity among bovine RVCs and low identity between human and porcine RVCs. Phylogenetic analysis of each gene showed that the two bovine RVCs belong to a cluster distinct from human and porcine RVCs. These data demonstrate that RVCs can be classified into different genotypes according to host species. Moreover, RVC NSP1, NSP2 and VP1 amino acid sequences contain a unique motif that is highly conserved among rotavirus A (RVA) strains and, hence, several proteins from bovine RVCs are suggested to play important roles that are similar to those of RVAs.

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INTRODUCTION

Rotavirus, one of major enteric pathogens of humans and other animals, is currently divided into eight species (A–H) on the basis of serological and genomic analyses (Estes & Kapikian, 2007; Matthijnssens *et al.*, 2012). The viral genome consists of 11 dsRNA segments encoding six structural proteins (VP1–4, VP6 and VP7) and five or six non-structural proteins (NSP1–6) (Jiang *et al.*, 1996). The viral particle is composed of three concentric layers. The outer capsid consists of two structural proteins, VP4 and VP7, which contain neutralization antigens. The inner capsid consists of the structural protein VP6. The core (inner layer) consists of VP2, the major inner core protein, VP1, the RNA-dependent RNA polymerase, and VP3, the viral capping enzyme. The NSPs are known to be involved in viral replication, transcription, cellular pathology and morphogenesis (Estes & Kapikian, 2007).

Rotavirus C (RVC) was first identified in swine as a causative agent of diarrhoea in the 1980s (Saif *et al.*, 1980; Bohl *et al.*, 1982). Subsequently, several reports showed an association between porcine RVC and diarrhoea in nursing, weaning and post-weaning pigs (Sigolo de San Juan *et al.*, 1986; Morin *et al.*, 1990; Saif & Jiang, 1994; Kim *et al.*, 1999). To date, RVCs have been detected in humans, cows, ferrets and dogs (Rodger *et al.*, 1982; Torres-Medina, 1987; Tsunemitsu *et al.*, 1991; Chang *et al.*, 1999; Otto *et al.*, 1999; Mawatari *et al.*, 2004). Human RVCs have been detected not only exclusively in children under 3 years old, but also in all age groups including adults in many countries (Peñaranda *et al.*, 1989; Kuzuya *et al.*, 1998; Matsumoto *et al.*, 1989; Qiao *et al.*, 1999; Nilsson *et al.*, 2000; Adah *et al.*, 2002; Castello *et al.*, 2002; Mwenda *et al.*, 2003; Sánchez-Fauquier *et al.*, 2003; Phan *et al.*, 2004; Schnagl *et al.*, 2004; Rahman *et al.*, 2005; Abid *et al.*, 2007;

The GenBank/EMBL/DDBJ accession numbers for the bovine RVC sequences determined in this study are AB738402–AB738417, as detailed in Fig. 1.

Three supplementary tables are available with the online version of this paper.

Esona *et al.*, 2008; Gabbay *et al.*, 2008; Medici *et al.*, 2009; Mitui *et al.*, 2009; Luchs *et al.*, 2011; Moon *et al.*, 2011). However, the prevalence of RVC in gastroenteric disease of children was reported to be relatively low, in spite of the global distribution of the virus (Mackow, 1995). Apart from RVC infections in humans, a high prevalence of antibodies in cattle and pigs (47–56 and 93–97 %, respectively) was detected in the United States and Japan (Tsunemitsu *et al.*, 1992). In contrast, another epidemiological study done in the United States showed that the detection rate of RVC in faecal samples from both diarrhoeic calves and adult cows using an RT-PCR method was very low (Chang *et al.*, 1999). Hence, there are discrepancies between previous reports and, furthermore, the potential zoonotic and economic impact of RVC in cows remains unclear because of the limited genetic data available for bovine RVCs.

Whole-genome analysis is beneficial for molecular characterization and understanding of the evolution of the pathogen. It is also useful for monitoring gene reassortment and interspecies transmission between different viral strains (Matthijnssens *et al.*, 2008a, b, 2010; McDonald *et al.*, 2009). Recently, a classification system for rotavirus A (RVA) was developed to differentiate all segments into genotypes (Matthijnssens *et al.*, 2008b). However, whole-genome analysis is to be preferred because of the difficulty in adapting RVCs to cell-culture propagation. To date, the full-genome sequence from one porcine RVC strain, Cowden, and several human RVC strains have been analysed, but those from bovine strains have not been fully elucidated (Mackow, 1995; Chen *et al.*, 2002; Yamamoto *et al.*, 2011). The bovine RVC strain Shintoku was isolated from four dairy cows (aged 28–41 months) affected with severe diarrhoea at a farm in Hokkaido Prefecture, Japan, in 1991 (Tsunemitsu *et al.*, 1991). Another bovine RVC strain, Toyama, was detected in dairy cows with sporadic diarrhoea in Toyama Prefecture, Japan, in 2010. To our knowledge, of several bovine RVCs, only the Shintoku strain could be serially propagated and maintained in a rhesus monkey kidney cell line (MA104) (Kusanagi *et al.*, 1992).

Genomic sequences of the bovine RVC Shintoku strain have already been determined for the VP3, VP4, VP6, VP7, NSP3 and NSP5 genes (Jiang, *et al.*, 1992, 1993; Tsunemitsu *et al.*, 1996). Sequence analyses of VP6 genes from bovine RVCs have been performed on only a few strains isolated in the United States (WD534tc) and Japan (Shintoku and Yamagata strains) (Jiang *et al.*, 1992; Chang *et al.*, 1999; Mawatari *et al.*, 2004). In addition, phylogenetic analyses of VP6 and VP7 genes indicate that the Shintoku and Yamagata strains belong to the same cluster, which is genetically distinct from human and porcine RVCs, whilst the WD534tc strain is classified into a porcine RVC cluster on the basis of genetic analysis of VP6 (Mawatari *et al.*, 2004). It therefore remains unclear whether there is genetic diversity among bovine RVC strains, since no other reports have appeared of the detection of RVCs from cattle. In order to verify the genetic divergence among bovine RVCs, whole-genome data of bovine RVC strains other than the Shintoku strain would need to be accumulated.

In the present study, we attempted to determine the nucleotide sequences of the five underresearched genes (NSP1, NSP2, NSP4, VP1 and VP2) from the Shintoku strain and the ORF sequences of all 11 RNA segments from another bovine RVC strain, designated Toyama. Moreover, phylogenetic analysis of individual segments was also performed to establish a genetic classification among RVCs from different host species and to investigate genetic diversity among bovine RVCs.

RESULTS AND DISCUSSION

When nucleotide sequences of individual RVC segments were aligned, each sequence at the 5′- and 3′-ends was highly conserved (Kobayashi *et al.*, 2003). In fact, the VP6 gene of the Shintoku strain was detected and analysed using a set of primers designed on the basis of 5′- and 3′-terminal sequences from the human RVC Bristol strain (Jiang *et al.*, 1992). Consequently, RT-PCR with several pairs of primers, designed by reference to both ends of individual segments from the human RVC Bristol strain and/or the porcine RVC Cowden strain, was used to determine the full-length nucleotide sequences of NSP1, NSP2, VP1 and VP2 from the bovine RVC Shintoku strain (Table S1, available in JGV Online). Because the nucleotide sequences at both ends of the NSP4 gene, which has enterotoxigenic potential, were different between the Shintoku and Bristol strains, only the ORF sequence of the Shintoku NSP4 gene could be elucidated (see Table 2). In addition, ORF sequences of the remaining segments, except for NSP4, VP6 and VP7, of another bovine RVC strain, Toyama, were determined using a set of primers designed by reference to those of the Shintoku strain (Tables S1 and S2). The sizes of the 11 RNA segments as well as the sizes of the 11 ORFs of the two bovine RVC strains, in addition to those of human and porcine RVC strains, are summarized in Table 1. The lengths of the deduced amino acid sequences of NSP2, NSP3, VP1, VP6 and VP7 from the bovine RVCs were identical to those of human RVCs. NSP1 and VP3 from the bovine RVCs were one or two amino acids longer than those of human RVCs. On the other hand, bovine RVC NSP4, NSP5, VP2 and VP4 were shorter than their human RVC homologues.

Nucleotide and deduced amino acid sequence identities of the ORFs of individual genes among bovine RVC strains and between human and porcine RVC strains are shown in Tables 2 and S3. The sequences of corresponding segments were highly conserved among bovine RVCs (88.7–98.4 % identity at the nucleotide level; 94.7–100 % identity at the amino acid level). Throughout the 11 gene segments, the bovine RVCs exhibited relatively low sequence identities to human RVCs (57.6–82.1 % at the nucleotide level; 54.7–89.1 % at the amino acid level) and porcine RVCs (56.5–82.6 % at the

Table 1. Full lengths of nucleotide and deduced amino acid sequences for individual genes among RVCs

Strain	NSP1		NSP2		NSP3		NSP4		NSP5	
	nt	aa	nt	aa	nt	aa	nt	aa	nt	aa
Shintoku	1273	395	1037	312	1350	402	ND	146	719	209
Toyama	ND	395	ND	312	ND	402	ND	146	ND	209
Bristol	1270	394	1037	312	1350	402	613	150	730	212
BK0830	1270	394	1037	312	1350	402	613	150	730	212
BS347	1270	394	1037	312	1350	402	613	150	730	212
OH567	1270	394	1037	312	1350	402	613	150	730	212
v508	1270	394	1037	312	1350	402	613	150	730	212
Wu82	1270	394	1037	312	1350	402	613	150	730	212
YNR001	1270	394	1037	312	1350	402	613	150	730	212
Cowden	1235	393	995	312	1348	402	613	150	693	210

Strain	VP1		VP2		VP3		VP4		VP6		VP7	
	nt	aa	nt	aa	nt	aa	nt	aa	nt	aa	nt	aa
Shintoku	3309	1090	2727	881	2166	695	2253	733	1352	395	1063	332
Toyama	3309	1090	ND	881	ND	695	2253	733	1352	395	1063	332
Bristol	3309	1090	2736	884	2166	693	2283	744	1353	395	1063	332
BK0830	3309	1090	2736	884	2166	693	2283	744	1353	395	1063	332
BS347	3309	1090	2736	884	2166	693	2283	744	1353	395	1063	332
OH567	3309	1090	2736	884	2166	693	2283	744	1353	395	1063	332
v508	3309	1090	2736	884	2166	693	2283	744	1353	395	1063	332
Wu82	3309	1090	2736	884	2166	693	2283	744	1353	395	1063	332
YNR001	3309	1090	2736	884	2166	693	2283	744	1353	395	1063	332
Cowden	3290	1082	2736	884	2145	692	2246	736	1352	395	1063	332

ND, Not determined (see text).

nucleotide level; 60.7–91.6 % at the amino acid level). Of the 11 segments, NSP4 exhibited the lowest identity between bovine and other RVCs (56.5–59.7 % at the nucleotide level; 54.7–62.7 % at the amino acid level). In contrast, NSP2, VP1, VP2 and VP6 sequences were relatively conserved between bovine and other RVCs (>78 % at the nucleotide level; >83 % at the amino acid level). Moreover, the VP6 and VP7 nucleotide sequences of the Yamagata strain, which was detected in Japan in 2004, showed high identity to the Shintoku strain. In contrast, the phylogenetic tree based on partial sequences of VP6 genes from bovine RVCs detected in South Korea (positions 1095–1303) indicated that bovine RVCs were more closely related to porcine RVCs (Park *et al.*, 2011). In addition, genetic analysis of the RVC VP7 gene revealed that the WD534tc strain is genetically more closely related to the porcine RVC Cowden strain than to either the Shintoku or Yamagata strain (Chang *et al.*, 1999). These data suggest that bovine RVCs that are distributed widely in the world have wide diversity, but bovine RVCs in Japan may have originated from a common ancestor and then co-circulated around Japan.

Phylogenetic analyses of the 11 segments of the two bovine RVC strains were performed in comparison with those of several human and porcine strains. The Shintoku and Toyama strains belong to the same cluster, which is distinct from human and porcine RVCs on all segments (Fig. 1). Subsequently, a genetic classification of each RVC segment was carried out on the basis of a cut-off value estimated according to the definitions of a previous report (Yamamoto *et al.*, 2011; see Methods). The cut-off values for genotyping of individual genes are summarized in Table 2. In the tree of the RVC VP7 gene, the Shintoku and Toyama strains were grouped into genotype G2 on the basis of the previous genetic classification (Martella *et al.*, 2007). In the genotyping of the RVC VP4 gene, the two bovine RVCs were classified into genotype P[3], whilst the human and porcine RVCs were grouped into genotypes P[2] and P[1], respectively. For the NSP3, NSP5, VP3 and VP6 genes, the two bovine RVCs were assigned to genotypes T3, H3, M4 and I3, respectively, on the pre-existing genotype classification (Yamamoto *et al.*, 2011) Phylogenetic analysis of the genes encoding NSP1, NSP2, NSP4, VP1 and VP2 indicated the presence of new genotypes harbouring the bovine RVCs, provisionally defined as A3, N3, E3, R3 and C3.

The presence of divergent or conserved regions in all segments among RVCs and between them and RVAs and/or RVBs was analysed by alignment of deduced amino acid sequences. Consequently, several motifs in NSP1, NSP2 and VP1 were highly conserved among RVAs, other RVCs and some RVBs (Fig. 2). In comparing NSP1 amino acid sequences from the two bovine RVCs with those of RVAs, a

Table 2. Identities of individual genes at the nucleotide level among RVCs

Nucleotide sequences of the bovine RVC strains, Shintoku and Toyama, were compared with one another or among three strains including the Yamagata strain in the case of VP6 and VP7. In addition, sequences of the two bovine RVC strains were also compared with human and porcine RVC strains as shown in Fig. 1.

Gene segment	Host	*n*	Nucleotide sequence identity (%)			Cut-off (%)
			Human	Porcine	Bovine	
NSP1	Human	10	92.8–99.9	66.3–67.8	66.3–67.7	74
	Porcine	1			73.2–73.7	74
	Bovine	2			96.0	74
NSP2	Human	10	93.7–100	85.2–88.0	80.6–82.1	89
	Porcine	1			82.5–82.6	89
	Bovine	2			98.4	89
NSP3	Human	10	92.6–99.8	76.3–78.2	77.5–79.2	80
	Porcine	1			77.3–77.7	80
	Bovine	2			97.2	80
NSP4	Human	13	94.6–99.8	68.8–70.3	57.6–59.7	71
	Porcine	1			56.5–57.9	71
	Bovine	2			93.2	71
NSP5	Human	10	92.0–99.5	76.0–76.8	74.6–75.9	79
	Porcine	1			78.1–78.5	79
	Bovine	2			95.3	79
VP1	Human	8	94.4–99.7	85.1–85.6	79.7–80.7	86
	Porcine	1			80.2–80.7	86
	Bovine	2			94.7	86
VP2	Human	9	94.5–99.8	82.7–83.3	78.8–79.3	84
	Porcine	1			80.2–80.4	84
	Bovine	2			95.9	84
VP3	Human	8	83.6–99.3	81.9–85.3	76.7–79.1	86
	Porcine	1			78.0–78.2	86
	Bovine	2			94.2	86
VP4	Human	13	95.4–99.7	71.9–72.7	70.3–70.9	77
	Porcine	1			75.9–76.3	77
	Bovine	2			97.0	77
VP6	Human	13	94.2–100	81.4–84.0	78.8–82.4	–
	Porcine	1			78.7–95.3	–
	Bovine	4			77.9–98.0	–
VP6 (exceptWD534tc)	Human	13	94.2–100	81.4–84.0	78.8–81.6	85
	Porcine	1			78.7–80.8	85
	Bovine	3			94.0–98.0	85
VP7	Human	23	88.4–99.6	69.4–83.7	68.8–74.6	84
	Porcine	4		71.8–80.9	68.4–75.4	84
	Bovine	3			88.7–92.4	84

common zinc-binding motif at the N-terminal domain, highly conserved in RVAs, was observed. This motif is known to be involved in evasion of the innate immune response to virus infection through binding to interferon regulatory factor 3 (IRF3) (Graff *et al.*, 2002). Therefore, the presence of the zinc-binding motif in bovine RVC NSP1 suggests that it is also involved in regulation of the host immune system, in the same manner as NSP1 from RVAs. The histidine triad motif (H$\varphi\varphi\varphi$H$\varphi\varphi$) of NSP2, which is important for NTPase activity, is highly conserved in RVAs, RVBs and other RVCs (Carpio *et al.*, 2004; Suzuki *et al.*, 2012). The fact that the bovine RVC NSP2 also possesses the histidine triad motif suggests that the motif is essential for the viral replication process among different species of rotaviruses. Moreover, the amino acid sequences of the VP1 gene from the two bovine RVCs contain the consensus motif of the RNA polymerase that is highly conserved among RNA viruses (Cohen *et al.*, 1989; Nagashima *et al.*, 2008). This finding suggests that the product of the bovine RVC VP1 gene functions as an RNA-dependent RNA polymerases, as is the case for other rotaviruses.

In conclusion, our study demonstrates that bovine RVCs can be classified into genotypes distinct from human and porcine RVCs according to genetic relatedness and the

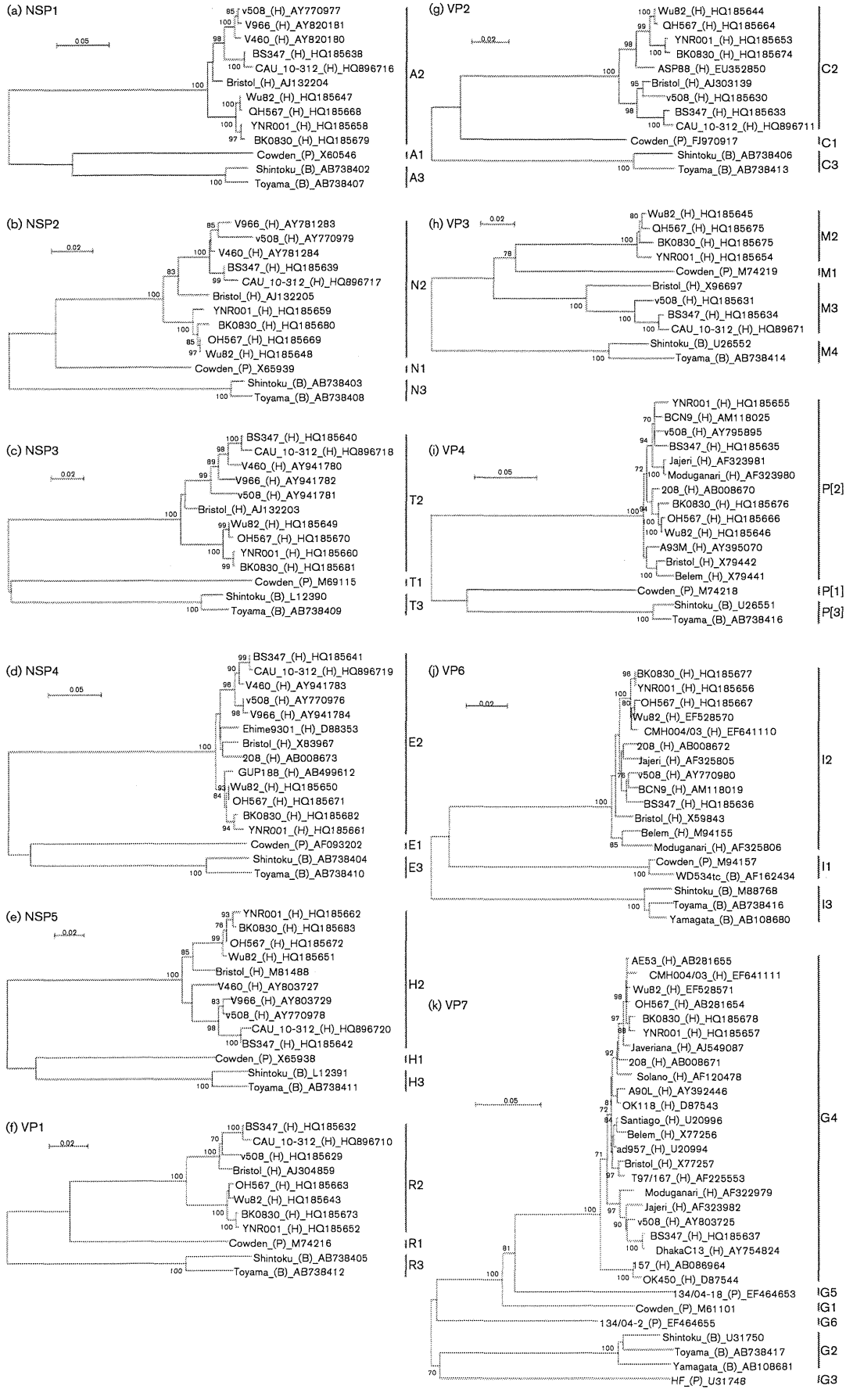
(a) NSP1
v508_(H)_AY770977
V966_(H)_AY820181
V460_(H)_AY820180
BS347_(H)_HQ185638
CAU_10-312_(H)_HQ896716
Bristol_(H)_AJ132204
Wu82_(H)_HQ185647
QH567_(H)_HQ185668
YNR001_(H)_HQ185658
BK0830_(H)_HQ185679
Cowden_(P)_X60546
Shintoku_(B)_AB738402
Toyama_(B)_AB738407
A2
A1
A3
(b) NSP2
V966_(H)_AY781283
v508_(H)_AY770979
V460_(H)_AY781284
BS347_(H)_HQ185639
CAU_10-312_(H)_HQ896717
Bristol_(H)_AJ132205
YNR001_(H)_HQ185659
BK0830_(H)_HQ185680
OH567_(H)_HQ185669
Wu82_(H)_HQ185648
Cowden_(P)_X65939
Shintoku_(B)_AB738403
Toyama_(B)_AB738408
N2
N1
N3
(c) NSP3
BS347_(H)_HQ185640
CAU_10-312_(H)_HQ896718
V460_(H)_AY941780
V966_(H)_AY941782
v508_(H)_AY941781
Bristol_(H)_AJ132203
Wu82_(H)_HQ185649
OH567_(H)_HQ185670
YNR001_(H)_HQ185660
BK0830_(H)_HQ185681
Cowden_(P)_M69115
Shintoku_(B)_L12390
Toyama_(B)_AB738409
T2
T1
T3
(d) NSP4
BS347_(H)_HQ185641
CAU_10-312_(H)_HQ896719
V460_(H)_AY941783
v508_(H)_AY770976
V966_(H)_AY941784
Ehime9301_(H)_D88353
Bristol_(H)_X83967
208_(H)_AB008673
GUP188_(H)_AB499612
Wu82_(H)_HQ185650
OH567_(H)_HQ185671
BK0830_(H)_HQ185682
YNR001_(H)_HQ185661
Cowden_(P)_AF093202
Shintoku_(B)_AB738404
Toyama_(B)_AB738410
E2
E1
E3
(e) NSP5
YNR001_(H)_HQ185662
BK0830_(H)_HQ185683
OH567_(H)_HQ185672
Wu82_(H)_HQ185651
Bristol_(H)_M81488
V460_(H)_AY803727
V966_(H)_AY803729
v508_(H)_AY770978
CAU_10-312_(H)_HQ896720
BS347_(H)_HQ185642
Cowden_(P)_X65938
Shintoku_(B)_L12391
Toyama_(B)_AB738411
H2
H1
H3
(f) VP1
BS347_(H)_HQ185632
CAU_10-312_(H)_HQ896710
v508_(H)_HQ185629
Bristol_(H)_AJ304859
OH567_(H)_HQ185663
Wu82_(H)_HQ185643
BK0830_(H)_HQ185673
YNR001_(H)_HQ185652
Cowden_(P)_M74216
Shintoku_(B)_AB738405
Toyama_(B)_AB738412
R2
R1
R3
(g) VP2
Wu82_(H)_HQ185644
QH567_(H)_HQ185664
YNR001_(H)_HQ185653
BK0830_(H)_HQ185674
ASP88_(H)_EU352850
Bristol_(H)_AJ303139
v508_(H)_HQ185630
BS347_(H)_HQ185633
CAU_10-312_(H)_HQ896711
Cowden_(P)_FJ970917
Shintoku_(B)_AB738406
Toyama_(B)_AB738413
C2
C1
C3
(h) VP3
Wu82_(H)_HQ185645
QH567_(H)_HQ185675
BK0830_(H)_HQ185675
YNR001_(H)_HQ185654
Cowden_(P)_M74219
Bristol_(H)_X96697
v508_(H)_HQ185631
BS347_(H)_HQ185634
CAU_10-312_(H)_HQ89671
Shintoku_(B)_U26552
Toyama_(B)_AB738414
M2
M1
M3
M4
(i) VP4
YNR001_(H)_HQ185655
BCN9_(H)_AM118025
v508_(H)_AY795895
BS347_(H)_HQ185635
Jajeri_(H)_AF323981
Moduganari_(H)_AF323980
208_(H)_AB008670
BK0830_(H)_HQ185676
OH567_(H)_HQ185666
Wu82_(H)_HQ185646
A93M_(H)_AY395070
Bristol_(H)_X79442
Belem_(H)_X79441
Cowden_(P)_M74218
Shintoku_(B)_U26551
Toyama_(B)_AB738416
P[2]
P[1]
P[3]
(j) VP6
BK0830_(H)_HQ185677
YNR001_(H)_HQ185656
OH567_(H)_HQ185667
Wu82_(H)_EF528570
CMH004/03_(H)_EF641110
208_(H)_AB008672
Jajeri_(H)_AF325805
v508_(H)_AY770980
BCN9_(H)_AM118019
BS347_(H)_HQ185636
Bristol_(H)_X59843
Belem_(H)_M94155
Moduganari_(H)_AF325806
Cowden_(P)_M94157
WD534tc_(B)_AF162434
Shintoku_(B)_M88768
Toyama_(B)_AB738416
Yamagata_(B)_AB108680
I2
I1
I3
(k) VP7
AE53_(H)_AB281655
CMH004/03_(H)_EF641111
Wu82_(H)_EF528571
OH567_(H)_AB281654
BK0830_(H)_HQ185678
YNR001_(H)_HQ185657
Javeriana_(H)_AJ549087
208_(H)_AB008671
Solano_(H)_AF120478
A90L_(H)_AY392446
OK118_(H)_D87543
Santiago_(H)_U20996
Belem_(H)_X77256
ad957_(H)_U20994
Bristol_(H)_X77257
T97/167_(H)_AF225553
Moduganari_(H)_AF322979
Jajeri_(H)_AF323982
v508_(H)_AY803725
BS347_(H)_HQ185637
DhakaC13_(H)_AY754824
157_(H)_AB086964
OK450_(H)_D87544
134/04-18_(P)_EF464653
Cowden_(P)_M61101
134/04-2_(P)_EF464655
Shintoku_(B)_U31750
Toyama_(B)_AB738417
Yamagata_(B)_AB108681
HF_(P)_U31748
G4
G5
G1
G6
G2
G3

Fig. 1. Phylogenetic trees for all individual segments from RVCs. The dendrograms were reconstructed by the neighbour-joining method with the MEGA 5 program. Percentages of bootstrap support (from 1000 replicates) are indicated at each node; values less than 70% are not indicated. B, Bovine; H, human; P, porcine. GenBank accession numbers are also shown. Genotypes are given to the right.

species of origin. The fact that the two bovine RVCs identified in Japan are genetically more closely related to each another suggests that bovine RVCs derived from a common origin are circulating among farms throughout Japan. The data presented here will expand molecular information and aid the progress of genetic analysis of RVCs.

METHODS

Viruses. The RVC Shintoku strain was propagated and maintained in MA104 cells in our laboratory according to a method described previously (Tsunemitsu *et al.*, 1991). The RVC Toyama strain originated from faecal samples of diarrhoeic dairy cows in 2010. Viral RNA was extracted from culture fluid or 10% faecal suspension diluted with PBS using TRIzol LS (Invitrogen) according to the manufacturer's instructions.

RT-PCR and sequencing. The full-length nucleotide sequences of four genes, NSP1, NSP2, VP1 and VP2, and the ORF sequence of the NSP4 gene from the Shintoku strain were amplified and sequenced by RT-PCR using primers originally designed to correspond to genes of the human RVC Bristol and/or porcine RVC Cowden strains (Table S1). The ORF sequences of the 11 RNA segments from the Toyama strain were amplified and sequenced by RT-PCR using primers designed to correspond to the genes of the Shintoku strain, in addition to primers used in the analysis of five unidentified genes of the Shintoku strain (Table S2). RT-PCRs were carried out using a OneStep RT-PCR kit (Qiagen) according to the following conditions: 50 °C for 30 min and 95 °C for 15 min, followed by 35 cycles of 94 °C for 1 min, 48 °C for 1 min and 72 °C for 2 min, and then a final extension at 72 °C for 10 min. The products were sequenced using a BigDye Terminator version 3.1 cycle sequencing kit on an automated ABI Prism 3130 Genetic Analyzer (Applied Biosystems).

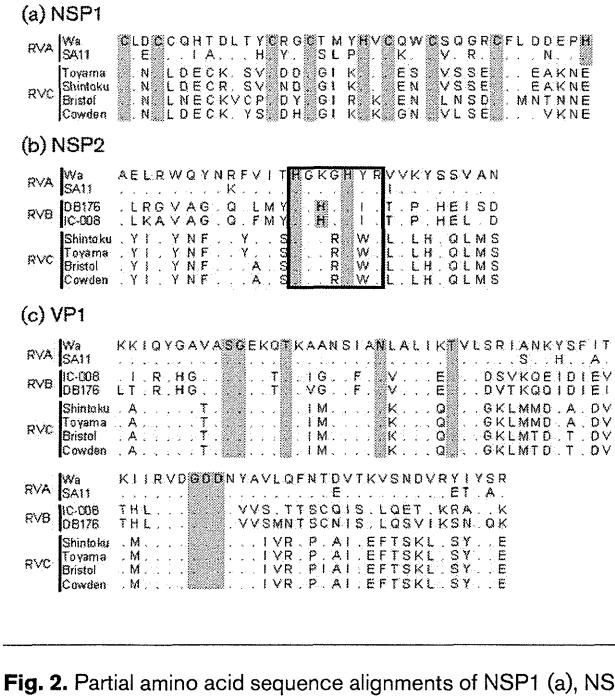

Fig. 2. Partial amino acid sequence alignments of NSP1 (a), NSP2 (b) and VP1 (c) from human RVA (Wa), simian RVA (SA11), human RVC (Bristol), porcine RVC (Cowden), two bovine RVCs (Shintoku and Toyama), human RVB (IC-008) and bovine RVB (DB176). Dots indicate amino acids identical to those of the human RVA Wa strain. (a) The zinc-finger motif and cysteine- and histidine-rich regions are shaded. (b) A histidine triad motif, an active site of nucleotide triphosphatase, is boxed. (c) The consensus motif of RNA polymerase (SG, T, N, T, GDD) is shaded.

Sequence data were aligned by the CLUSTAL W method using the MEGALIGN 7.2 program of the Lasergene software (DNASTAR). Phylogenetic analyses were conducted using MEGA version 5 (Tamura *et al.*, 2011). Genetic distances were calculated using the Poisson correction parameter at the nucleotide level and Kimura's two-parameter correction at the amino acid level (Matthijnssens *et al.*, 2008b). Phylogenetic trees were constructed with 1000 bootstrap replicates (Saitou & Nei, 1987).

In order to classify individual genotypes of each of the bovine RVC segments, a cut-off value was determined according to the methods described by Yamamoto *et al.* (2011). Briefly, the cut-off value for genotyping of each gene segment was defined as a value that is slightly higher than the maximum sequence identity at the nucleotide level between human and animal RVCs, because these RVCs were grouped into different clusters in the phylogenetic dendrograms as shown in Fig. 1. The values for genetic classification among RVCs including the two bovine RVC strains, Shintoku and Toyama, are summarized in Table 2.

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REFERENCES

Abid, I., Guix, S., Aouni, M., Pintó, R. & Bosch, A. (2007). Detection and characterization of human group C rotavirus in the pediatric population of Barcelona, Spain. *J Clin Virol* **38**, 78–82.

Adah, M. I., Wade, A., Oseto, M., Kuzuya, M. & Taniguchi, K. (2002). Detection of human group C rotaviruses in Nigeria and sequence analysis of their genes encoding VP4, VP6, and VP7 proteins. *J Med Virol* **66**, 269–275.

Bohl, E. H., Saif, L. J., Theil, K. W., Agnes, A. G. & Cross, R. F. (1982). Porcine pararotavirus: detection, differentiation from rotavirus, and pathogenesis in gnotobiotic pigs. *J Clin Microbiol* **15**, 312–319.

Carpio, R. V., González-Nilo, F. D., Jayaram, H., Spencer, E., Prasad, B. V., Patton, J. T. & Taraporewala, Z. F. (2004). Role of the histidine triad-like motif in nucleotide hydrolysis by the rotavirus RNA-packaging protein NSP2. *J Biol Chem* **279**, 10624–10633.

Castello, A. A., Argüelles, M. H., Villegas, G. A., Olthoff, A. & Glikmann, G. (2002). Incidence and prevalence of human group C rotavirus infections in Argentina. *J Med Virol* **67**, 106–112.

Chang, K. O., Nielsen, P. R., Ward, L. A. & Saif, L. J. (1999). Dual infection of gnotobiotic calves with bovine strains of group A and porcine-like group C rotaviruses influences pathogenesis of the group C rotavirus. *J Virol* **73**, 9284–9293.

Chen, Z., Lambden, P. R., Lau, J., Caul, E. O. & Clarke, I. N. (2002). Human group C rotavirus: completion of the genome sequence and gene coding assignments of a non-cultivatable rotavirus. *Virus Res* **83**, 179–187.

Cohen, J., Charpilienne, A., Chilmonczyk, S. & Estes, M. K. (1989). Nucleotide sequence of bovine rotavirus gene 1 and expression of the gene product in baculovirus. *Virology* **171**, 131–140.

Esona, M. D., Humphrey, C. D., Dennehy, P. H. & Jiang, B. (2008). Prevalence of group C rotavirus among children in Rhode Island, United States. *J Clin Virol* **42**, 221–224.

Estes, M. K. & Kapikian, A. Z. (2007). Rotaviruses. In *Fields Virology*, 5th edn, pp. 1917–1974. Edited by D. M. Knipe, P. M. Howley, D. E. Griffin, R. A. Lamb, M. A. Martin, B. Roizman & S. E. Straus. Philadelphia: Wolters Kluwer Health/Lippincott Williams & Wilkins.

Gabbay, Y. B., Borges, A. A., Oliveira, D. S., Linhares, A. C., Mascarenhas, J. D., Barardi, C. R., Simões, C. M., Wang, Y., Glass, R. I. & Jiang, B. (2008). Evidence for zoonotic transmission of group C rotaviruses among children in Belém, Brazil. *J Med Virol* **80**, 1666–1674.

Graff, J. W., Mitzel, D. N., Weisend, C. M., Flenniken, M. L. & Hardy, M. E. (2002). Interferon regulatory factor 3 is a cellular partner of rotavirus NSP1. *J Virol* **76**, 9545–9550.

Jiang, B., Tsunemitsu, H., Gentsch, J. R., Glass, R. I., Green, K. Y., Qian, Y. & Saif, L. J. (1992). Nucleotide sequence of gene 5 encoding the inner capsid protein (VP6) of bovine group C rotavirus: comparison with corresponding genes of group C, A, and B rotaviruses. *Virology* **190**, 542–547.

Jiang, B., Tsunemitsu, H., Gentsch, J. R., Saif, L. J. & Glass, R. I. (1993). Nucleotide sequences of genes 6 and 10 of a bovine group C rotavirus. *Nucleic Acids Res* **21**, 2250.

Jiang, B., Tsunemitsu, H., Dennehy, P. H., Oishi, I., Brown, D., Schnagl, R. D., Oseto, M., Fang, Z. Y., Avendano, L. F. & other authors (1996). Sequence conservation and expression of the gene encoding the outer capsid glycoprotein among human group C rotaviruses of global distribution. *Arch Virol* **141**, 381–390.

Kim, Y., Chang, K. O., Straw, B. & Saif, L. J. (1999). Characterization of group C rotaviruses associated with diarrhea outbreaks in feeder pigs. *J Clin Microbiol* **37**, 1484–1488.

Kobayashi, N., Alam, M., Kojima, K., Mise, K., Ishino, M. & Sumi, A. (2003). Genomic diversity and evolution of rotaviruses: an overview. In *Genomic Diversity and Molecular Epidemiology of Rotaviruses*, pp. 75–90. Edited by N. Kobayashi. Trivandrum, India: Research Signpost.

Kusanagi, K., Kuwahara, H., Katoh, T., Nunoya, T., Ishikawa, Y., Samejima, T. & Tajima, M. (1992). Isolation and serial propagation of porcine epidemic diarrhea virus in cell cultures and partial characterization of the isolate. *J Vet Med Sci* **54**, 313–318.

Kuzuya, M., Fujii, R., Hamano, M., Yamada, M., Shinozaki, K., Sasagawa, A., Hasegawa, S., Kawamoto, H., Matsumoto, K. & other authors (1998). Survey of human group C rotaviruses in Japan during the winter of 1992 to 1993. *J Clin Microbiol* **36**, 6–10.

Luchs, A., Morillo, S. G., de Oliveira, C. M. & Timenetsky, M. C. (2011). Monitoring of group C rotavirus in children with acute gastroenteritis in Brazil: an emergent epidemiological issue after rotavirus vaccine? *J Med Virol* **83**, 1631–1636.

Mackow, E. R. (1995). Group B and C rotaviruses. In *Infections of the Gastrointestinal Tract*, pp. 983–1008. Edited by M. J. Blaser, P. D. Smith, J. I. Ravdin, H. B. Greenberg & R. L. Guerrant. New York: Raven Press.

Martella, V., Bányai, K., Lorusso, E., Decaro, N., Bellacicco, A., Desario, C., Corrente, M., Greco, G., Moschidou, P. & other authors (2007). Genetic heterogeneity in the VP7 of group C rotaviruses. *Virology* **367**, 358–366.

Matsumoto, K., Hatano, M., Kobayashi, K., Hasegawa, A., Yamazaki, S., Nakata, S., Chiba, S. & Kimura, Y. (1989). An outbreak of gastroenteritis associated with acute rotaviral infection in schoolchildren. *J Infect Dis* **160**, 611–615.

Matthijnssens, J., Ciarlet, M., Rahman, M., Attoui, H., Bányai, K., Estes, M. K., Gentsch, J. R., Iturriza-Gómara, M., Kirkwood, C. D. & other authors (2008a). Recommendations for the classification of group A rotaviruses using all 11 genomic RNA segments. *Arch Virol* **153**, 1621–1629.

Matthijnssens, J., Ciarlet, M., Heiman, E., Arijs, I., Delbeke, T., McDonald, S. M., Palombo, E. A., Iturriza-Gómara, M., Maes, P. & other authors (2008b). Full genome-based classification of rotaviruses reveals a common origin between human Wa-like and porcine rotavirus strains and human DS-1-like and bovine rotavirus strains. *J Virol* **82**, 3204–3219.

Matthijnssens, J., Taraporewala, Z. F., Yang, H., Rao, S., Yuan, L., Cao, D., Hoshino, Y., Mertens, P. P., Carner, G. R. & other authors (2010). Simian rotaviruses possess divergent gene constellations that originated from interspecies transmission and reassortment. *J Virol* **84**, 2013–2026.

Matthijnssens, J., Otto, P. H., Ciarlet, M., Desselberger, U., Van Ranst, M. & Johne, R. (2012). VP6-sequence-based cutoff values as a criterion for rotavirus species demarcation. *Arch Virol* **157**, 1177–1182.

Mawatari, T., Taneichi, A., Kawagoe, T., Hosokawa, M., Togashi, K. & Tsunemitsu, H. (2004). Detection of a bovine group C rotavirus from adult cows with diarrhea and reduced milk production. *J Vet Med Sci* **66**, 887–890.

McDonald, S. M., Matthijnssens, J., McAllen, J. K., Hine, E., Overton, L., Wang, S., Lemey, P., Zeller, M., Van Ranst, M. & other authors (2009). Evolutionary dynamics of human rotaviruses: balancing reassortment with preferred genome constellations. *PLoS Pathog* **5**, e1000634.

Medici, M. C., Abelli, L. A., Martinelli, M., Martella, V., Dettori, G. & Chezzi, C. (2009). Molecular characterization of group C rotaviruses detected in children in Italy. *J Clin Virol* **44**, 62–65.

Mitui, M. T., Bozdayi, G., Dalgic, B., Bostanci, I., Nishizono, A. & Ahmed, K. (2009). Molecular characterization of a human group C rotavirus detected first in Turkey. *Virus Genes* **39**, 157–164.

Moon, S., Humphrey, C. D., Kim, J. S., Baek, L. J., Song, J. W., Song, K. J. & Jiang, B. (2011). First detection of group C rotavirus in children with acute gastroenteritis in South Korea. *Clin Microbiol Infect* **17**, 244–247.

Morin, M., Magar, R. & Robinson, Y. (1990). Porcine group C rotavirus as a cause of neonatal diarrhea in a Quebec swine herd. *Can J Vet Res* **54**, 385–389.

Mwenda, J. M., Peenze, I., Omollo, E., Galo, M. & Steele, A. D. (2003). Human group C rotaviruses identified in Kenya. *East Afr Med J* **80**, 73–76.

Nagashima, S., Kobayashi, N., Ishino, M., Alam, M. M., Ahmed, M. U., Paul, S. K., Ganesh, B., Chawla-Sarkar, M., Krishnan, T. & other authors (2008). Whole genomic characterization of a human rotavirus strain B219 belonging to a novel group of the genus Rotavirus. *J Med Virol* **80**, 2023–2033.

Nilsson, M., Svenungsson, B., Hedlund, K. O., Uhnoo, I., Lagergren, A., Akre, T. & Svensson, L. (2000). Incidence and genetic diversity of group C rotavirus among adults. *J Infect Dis* **182**, 678–684.

Otto, P., Schulze, P. & Herbst, W. (1999). Demonstration of group C rotaviruses in fecal samples of diarrheic dogs in Germany. *Arch Virol* **144**, 2467–2473.

Park, S. I., Jeong, Y. J., Kim, H. J., Park, J. G., Kang, S. Y., Woo, S. K., Kim, C. H., Jung, C. H., Kang, M. I. & Cho, K. O. (2011). Genetically diverse group C rotaviruses cause sporadic infection in Korean calves. *J Vet Med Sci* **73**, 479–482.

Peñaranda, M. E., Cubitt, W. D., Sinarachatanant, P., Taylor, D. N., Likanonsakul, S., Saif, L. & Glass, R. I. (1989). Group C rotavirus infections in patients with diarrhea in Thailand, Nepal, and England. *J Infect Dis* **160**, 392–397.

Phan, T. G., Nishimura, S., Okame, M., Nguyen, T. A., Khamrin, P., Okitsu, S., Maneekarn, N. & Ushijima, H. (2004). Virus diversity and an outbreak of group C rotavirus among infants and children with diarrhea in Maizuru city, Japan during 2002–2003. *J Med Virol* **74**, 173–179.

Qiao, H., Nilsson, M., Abreu, E. R., Hedlund, K. O., Johansen, K., Zaori, G. & Svensson, L. (1999). Viral diarrhea in children in Beijing, China. *J Med Virol* **57**, 390–396.

Rahman, M., Banik, S., Faruque, A. S., Taniguchi, K., Sack, D. A., Van Ranst, M. & Azim, T. (2005). Detection and characterization of human group C rotaviruses in Bangladesh. *J Clin Microbiol* **43**, 4460–4465.

Rodger, S. M., Bishop, R. F. & Holmes, I. H. (1982). Detection of a rotavirus-like agent associated with diarrhea in an infant. *J Clin Microbiol* **16**, 724–726.

Saif, L. J. & Jiang, B. (1994). Nongroup A rotaviruses of humans and animals. *Curr Top Microbiol Immunol* **185**, 339–371.

Saif, L. J., Bohl, E. H., Theil, K. W., Cross, R. F. & House, J. A. (1980). Rotavirus-like, calicivirus-like, and 23-nm virus-like particles associated with diarrhea in young pigs. *J Clin Microbiol* **12**, 105–111.

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

Sánchez-Fauquier, A., Roman, E., Colomina, J., Wilhelmi, I., Glass, R. I. & Jiang, B. (2003). First detection of group C rotavirus in children with acute diarrhea in Spain. *Arch Virol* **148**, 399–404.

Schnagl, R. D., Boniface, K., Cardwell, P., McCarthy, D., Ondracek, C., Coulson, B., Erlich, J. & Morey, F. (2004). Incidence of group C human rotavirus in central Australia and sequence variation of the VP7 and VP4 genes. *J Clin Microbiol* **42**, 2127–2133.

Sigolo de San Juan, C., Bellinzoni, R. C., Mattion, N., La Torre, J. & Scodeller, E. A. (1986). Incidence of group A and atypical rotaviruses in Brazilian pig herds. *Res Vet Sci* **41**, 270–272.

Suzuki, T., Soma, J., Kuga, K., Miyazaki, A. & Tsunemitsu, H. (2012). Sequence and phylogenetic analyses of nonstructural protein 2 genes of species B porcine rotaviruses detected in Japan during 2001–2009. *Virus Res* **165**, 46–51.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M. & Kumar, S. (2011). MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol Biol Evol* **28**, 2731–2739.

Torres-Medina, A. (1987). Isolation of an atypical rotavirus causing diarrhea in neonatal ferrets. *Lab Anim Sci* **37**, 167–171.

Tsunemitsu, H., Saif, L. J., Jiang, B. M., Shimizu, M., Hiro, M., Yamaguchi, H., Ishiyama, T. & Hirai, T. (1991). Isolation, characterization, and serial propagation of a bovine group C rotavirus in a monkey kidney cell line (MA104). *J Clin Microbiol* **29**, 2609–2613.

Tsunemitsu, H., Jiang, B. & Saif, L. J. (1992). Detection of group C rotavirus antigens and antibodies in animals and humans by enzyme-linked immunosorbent assays. *J Clin Microbiol* **30**, 2129–2134.

Tsunemitsu, H., Jiang, B. & Saif, L. J. (1996). Sequence comparison of the VP7 gene encoding the outer capsid glycoprotein among animal and human group C rotaviruses. *Arch Virol* **141**, 705–713.

Yamamoto, D., Ghosh, S., Kuzuya, M., Wang, Y. H., Zhou, X., Chawla-Sarkar, M., Paul, S. K., Ishino, M. & Kobayashi, N. (2011). Whole-genome characterization of human group C rotaviruses: identification of two lineages in the VP3 gene. *J Gen Virol* **92**, 361–369.

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ORIGINAL ARTICLE

Development and application of one-step multiplex reverse transcription PCR for simultaneous detection of five diarrheal viruses in adult cattle

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Abstract A one-step multiplex reverse transcription (RT)-PCR method was developed for the simultaneous detection of five viruses causing diarrhea in adult cattle: bovine group A rotavirus (GAR), bovine group B rotavirus (GBR), bovine group C rotavirus (GCR), bovine coronavirus (BCV), and bovine torovirus (BToV). The detection limit of the one-step multiplex RT-PCR for GAR, GCR, BCV, and BToV was 10^2, 10^0, 10^1, and 10^2 $TCID_{50}$/ml, respectively, and that for GBR was 10^6 copies/ml. The one-step multiplex RT-PCR with newly designed primers to detect GAR had higher sensitivity than a single RT-PCR with conventional primers, with no false-positive reactions observed for ten other kinds of bovine RNA viruses To assess its field applicability, 59 of 60 fecal samples containing one of these five viruses from all 25 epidemic diarrhea outbreaks in adult cattle were positive in the one-step multiplex RT-PCR assay. Furthermore, using four additional fecal samples containing two viruses (GBR and BCV or BToV), two amplified products of the expected sizes were obtained simultaneously. In contrast, all 80 fecal samples lacking the five target viruses from normal adult cattle were negative in the multiplex assay. Taken together, our results indicate that the one-step multiplex RT-PCR developed here for the detection of GAR, GBR, GCR, BCV, and BToV can be expected to be a useful tool for the rapid and cost-effective diagnosis and surveillance of viral diarrhea in adult cattle.

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Introduction

Epidemic outbreaks of diarrhea frequently occur in adult cattle, resulting in major economic losses to the dairy industry due to rapid decreases in milk production by affected cattle. Bovine coronavirus (BCV) is a primary cause of adult cattle diarrhea, including winter dysentery (WD), which is characterized by the sudden onset of epidemic diarrhea, which occasionally presents with bloody feces and reduced milk production during the winter season [15, 32, 36, 44]. In addition, bovine group A rotavirus (GAR) [14, 38], bovine group B rotavirus (GBR) [7, 18, 45], bovine group C rotavirus (GCR) [28, 42], and bovine torovirus (BToV) [19, 22] also have been identified in field cases of adult cattle diarrhea. However, the frequency of adult cattle diarrhea caused by these viruses remains unclear.