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Isolation of an Avianlike Group A Rotavirus from a Calf with Diarrhea

HARALD BRÜSSOW,[1]* OSAMU NAKAGOMI,[2] GIUSEPPE GERNA,[3] AND WERNER EICHHORN[4]

Nestlé Research Centre, Nestec Ltd., Vers-chez-les Blanc, CH-1000 Lausanne 26, Switzerland[1]; Akita University School of Medicine, Hondo, Akita, Japan[2]; Virus Laboratory, Institute of Infectious Diseases, University of Pavia, IRCCS Policlinico San Matteo, 27100 Pavia, Italy[3]; and Veterinary Faculty, Institute of Medical Microbiology, Infectious and Epidemic Diseases, University of Munich, Munich, Germany[4]

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An atypical group A rotavirus (993/83) was isolated from a 3-day-old German calf with diarrhea. It differed from 35 conventional German bovine rotavirus isolates analyzed previously with respect to subgroup (strain 993/83 was non-subgroup I and non-subgroup II), serotype (strain 993/83 showed a two-way cross-reaction with serotype 7 and a one-way cross-reaction with serotype 3), and electropherotype (strain 993/83 showed comigrating gene segments 10 and 11). Isolate 993/83 reacted with only one of four monoclonal antibodies that recognized a common VP6 epitope(s). In addition, VP6 and VP2 of isolate 993/83 showed one-dimensional peptide maps that differed substantially from the peptide maps of VP6 and VP2 from all bovine rotavirus isolates. By RNA-RNA hybridization, the 993/83 probe failed to react with a panel of mammalian rotavirus strains, including bovine rotaviruses. It hybridized, however, to genomic RNA of an avian rotavirus strain. Isolate 993/83 could thus represent a candidate for a natural interspecies transmission of rotavirus between different classes of vertebrates.

Rotaviruses are the major etiological agents of acute diarrhea for infants, as well as for young animals of many other mammalian (e.g., monkey, cow, pig, sheep, horse, rabbit, mouse, dog, and cat) and avian (e.g., chicken, turkey, and pigeon) species (17). Experimentally, human rotaviruses can infect animals and induce diarrheal illness (22, 41). Conversely, animal rotaviruses can infect humans, as has been observed in vaccine trials with live attenuated bovine and simian rotavirus vaccines given to infants (18). Rotaviruses can thus cross species barriers. However, with the exceptions of one felinelike rotavirus isolated from a child with gastroenteritis (33) and two bovinelike rotaviruses isolated from two children with gastroenteritis (12a), there is not much documented evidence that such an interspecies transmission has actually occurred in nature. In the present report, we describe a rotavirus isolate from the feces of a 3-day-old calf with diarrhea which differed substantially from the known bovine rotavirus strains. However, serological and molecular analyses revealed a close relationship with an avian rotavirus.

MATERIALS AND METHODS

History of rotavirus 993/83 isolation. Rotavirus 993/83 was isolated from the stool sample of a 3-day-old dairy calf living in 1983 on a farm near Giessen, Germany. The calf had diarrhea for about half a day before the stool sample was collected. We do not know whether the calf received colostrum. This stool sample was sent by the local veterinarian to the Veterinary Faculty of the University of Giessen, Giessen, Germany, for diagnosis. Rotaviruses were detected by electron microscopy, whereas coronaviruses were not detected. Salmonellosis was ruled out by the bacteriology laboratory. The University of Giessen collaborated at the time with the Veterinary Faculty of the University of Munich on a survey of bovine rotaviruses, and therefore, the stool sample was sent to the Munich laboratory. There, a peculiar RNA electropherotype was detected in the stool sample (8), and the virus was adapted to cell culture growth by standard methods (1).

Viruses. Tables 1 and 2 list the rotavirus strains used in this study and their relevant characteristics. In addition, 35 cell culture-adapted bovine rotavirus strains obtained from a survey of isolates from Germany were used as references (3).

Protein electrophoresis. MA104 cells were labeled throughout the entire infection cycle with [^{35}S]methionine (15 μCi/ml; Dupont, NEN Research Products) in infection medium containing 1/10 the normal methionine content.

After destruction of the cell monolayers, rotavirus particles were recovered from the cell-free culture supernatant by high-speed centrifugation (90,000 × *g*, 2 h, 4°C) through a 20% sucrose cushion. The crude virus pellet was then purified by CsCl equilibrium centrifugation on preformed CsCl gradients (1.25 to 1.42 g/ml). Rotavirus particles banding at 1.36 g/ml (double-shelled particles) and at 1.38 g/ml (single-shelled particles) were recovered from the CsCl gradients with a Pasteur pipette, diluted with TNC (50 mM Tris hydrochloride [pH 7.5] containing 150 mM NaCl and 10 mM $CaCl_2$), and pelleted by a further high-speed centrifugation step (130,000 × *g*, 1 h, 4°C).

Protein electrophoresis was done with slab gels by the method of Laemmli (20), with 13% running gels and 3% stacking gels. Gels were fluorographed with Enlightning (Dupont, NEN), dried, and exposed on X-ray films (YAR-5; Eastman Kodak Co.).

The method of limited-proteolysis analysis followed exactly the digestion procedure for proteins in gel slices described by Cleveland et al. (6). A 10-μl amount of *Staphylococcus aureus* V8 protease (type XVII; Sigma Chemical

* Corresponding author.

TABLE 1. Relevant characteristics of the rotavirus strains used in the present study

Strain	Source	Serotype	Subgroup	Reference(s)
Wa	Human	1	II	40
Ku	Human	1	II	40
S-2	Human	2	I	40
KUN	Human	2	I	19
AU-1	Human	3	I	30
Hochi	Human	4	II	40
WI61	Human	9	II	5
FRV-1	Cat	3	I	33
Cat 97	Cat	3	I	2
RS 15	Dog	3	I	27
BI	Horse	3	—[a]	16
SA11	Vervet monkey	3	I	15
RRV	Rhesus monkey	3	I	15
OSU	Pig	5	I	15
NCDV	Bovine	6	I	15
678	Bovine	8	I	37
B223	Bovine	10	I	37
V1005	Bovine	10	I	4
KK3	Bovine	10	I	28, 37
PO-13	Pigeon	7	Non-I, non-II	26

[a] Unknown.

Co.) at a concentration of 1 μg/μl was added to each slot. V8 protease cleaves at the COOH-terminal side of aspartic and glutamic acid residues (6).

Electron microscopy. The viral particles were deposited on copper grids coated with a Formvar film and carbon. They were negatively stained with a 2% solution of phosphotungstic acid (pH 7.0). Particles were viewed with a Philips EM 300 electron microscope at 80-kV accelerating voltage.

RNA electrophoresis. Viral RNA was extracted from CsCl gradient-purified rotavirus particles by the phenol-chloroform method and concentrated by ethanol precipitation. The purified RNA was analyzed on 10 or 5% polyacrylamide gels prepared by the method of Laemmli (20) by using a 3% stacking gel. The gels were run in Tris-HCl (pH 8.0)-glycine-sodium dodecyl sulfate (SDS) buffer for 12 h at 40 V and 15 mA. The gels were stained with ethidium bromide. The photographic negatives were analyzed with a laser densitometer (LKB 2202 Ultrascan) coupled with a computing integrator.

Hyperimmunization of guinea pigs. Purified viral particles were emulsified in Freund's complete adjuvant (first injection) and in Freund's incomplete adjuvant (second injection), suspended in phosphate-buffered saline (PBS) (third injection), and administered intramuscularly into guinea pigs. Animals had been shown to be free of neutralizing antibodies to bovine rotaviruses UK and V1005 (4) and simian rotavirus SA11 (preimmune titers, <50). Sera were analyzed 20 days after the last injection for neutralizing activity against 100 50% tissue culture infective doses of the indicated rotavirus by using the immunoperoxidase focus reduction test of Gerna et al. (11). Neutralizing titers are expressed as the reciprocal of the serum dilution reducing the number of infected cells by 50%.

Enzyme-linked immunosorbent assay (ELISA). Microtiter plates were coated with purified single-shelled and EDTA-treated double-shelled rotavirus particles. Polyclonal mouse serum raised against bovine rotavirus UK, subgroup I- or subgroup II-specific monoclonal antibodies (14), or four VP6-specific monoclonal antibodies (12) were added at a 1:300 dilution in PBS–0.05% Tween 20. Bound antibody was revealed with affinity-purified antibody to mouse immunoglobulins coupled to alkaline phosphatase (Sigma). Sigma substrate 104 was used according to the manufacturer's instructions.

RNA-RNA hybridization in solution. RNA-RNA hybridization in solution was performed as previously described (29). Briefly, to denatured genomic RNA (100°C, 2 min) were added the ^{32}P-labeled probes (20,000 cpm for each denatured double-stranded RNA), which were prepared by in vitro transcription of rotavirus genomic RNA in the presence of [^{32}P]GTP. Hybridization was then allowed to occur at 65°C for 16 h in a buffer containing 5 mM Tris acetate, 150 mM NaCl, 1 mM EDTA, and 1% SDS (pH 7.5).

The resulting hybrids were fractionated on a 10% polyacrylamide gel. The gels were stained with ethidium bromide and then exposed to X-Omat AR films (Eastman Kodak Co.).

TABLE 2. Reassortants used in the present study

Reassortant	Origin of [strain (serotype)]:		Reference
	VP4	VP7	
S-4	SA11 (3)	NCDV (6)	35
N-4	NCDV (6)	SA11 (3)	35
DS-1 × RRV	RRV (3)	DS-1 (human, 2)	23
ST3 × RRV	RRV (3)	ST3 (human, 4)	24

RESULTS

Electropherotypes. The electropherotype of genomic RNA of isolate 993/83 differed from the electropherotypes of RNAs of conventional mammalian rotavirus strains in that segment 1 moved fast and migrated close to segment 2. In addition, segments 5 and 6 were widely spaced, and finally, only one segment which migrated between gene segments 10 and 11 of human rotavirus Wa could be resolved (Fig. 1). Densitometric analysis, however, confirmed the presence of two comigrating gene segments (Fig. 2). Gene segments 10 and 11 of SA11 rotavirus represent 4.0 and 3.6%, respectively, of the total genome size (9). In good agreement, 3.5 and 3.3% of the total genomic RNA fluorescence were detected in gene segments 10 and 11, respectively, of a typical bovine rotavirus (Fig. 2). In contrast, the smallest gene segment of isolate 993/83 showed 7.4% of the total genomic RNA fluorescence, which is twice the fluorescence expected for an RNA segment of this molecular weight. Also, we could not resolve the two gene segments 10 and 11 on 5% polyacrylamide gels.

Polypeptide composition. CsCl density gradient centrifugation of isolate 993/83 grown on MA104 cells resulted in the formation of a single band at a buoyant density of 1.36 g/ml. This band showed typical double-shelled rotavirus particles by electron microscopy (Fig. 3). SDS-polyacrylamide gel electrophoresis (PAGE) revealed that VP2 and VP6 of isolate 993/83 had lower electrophoretic mobilities than VP2 and VP6 of bovine rotaviruses did (Fig. 4). This mobility is typical for bovine rotaviruses, as it has been seen in another 20 bovine rotavirus isolates (data not shown). Interestingly, the one-dimensional peptide map of VP6 from isolate 993/83 differed markedly from the peptide maps of VP6 from all other bovine rotavirus isolates tested in our laboratory (Fig. 5B and data not shown). Similarly, the one-dimensional peptide map of VP2 from isolate 993/83 differed from that of VP2 from all bovine rotavirus isolates tested (Fig. 5A).

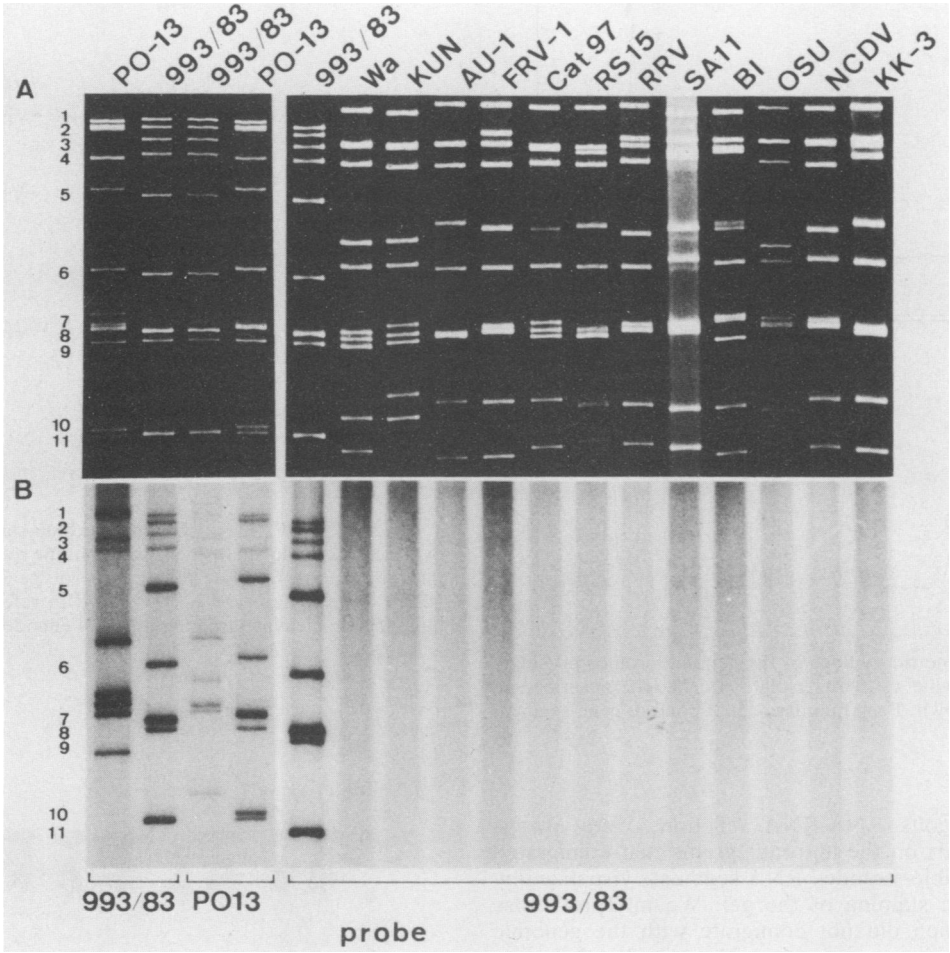

FIG. 1. Patterns of hybridization between genomic RNAs from the indicated virus strains and the ^{32}P-labeled transcription probe made from 993/83 and PO-13 rotavirus. (A) Ethidium bromide-stained gels under UV illumination; (B) corresponding autoradiogram. Approximate positions of the genomic RNA segments of rotavirus 993/83 are indicated at the left. The major characteristics of the rotaviruses used in this hybridization experiment are given in Table 1. Note that the extra bands in the leftmost lane of the ethidium bromide-stained gel containing PO-13 RNA (one band just above segment 6, three bands between segments 6 and 7, and one band just below segment 9) correspond to the bands seen in the autoradiogram. Similar extra bands obtained from hybridization of 993/83 RNA and the PO-13 probe (in the third lanes from the left) were also seen on the original photograph, but they disappeared during reproduction.

ELISA. Isolate 993/83 reacted with a mouse hyperimmune serum raised to bovine rotavirus UK in ELISA, which identified this isolate as a group A rotavirus, but it did not react with subgroup I- or subgroup II-specific monoclonal antibodies (Table 3). Isolate 993/83 also did not react with two broadly cross-reacting VP6-specific monoclonal antibodies (5D7 and 5C6) and reacted only weakly with a third VP6-specific monoclonal antibody (4B4). However, it shared with other group A rotaviruses an epitope on VP6, defined by monoclonal antibody 5A8 (Table 3). An identical reactivity pattern was observed for avian rotavirus PO-13 (Table 3). On the other hand, all bovine rotavirus isolates from Germany analyzed previously (3) belonged to subgroup I and reacted with all VP6-specific monoclonal antibodies (data not shown).

Neutralization. Isolate 993/83 was not significantly neutralized by animal hyperimmune sera to serotype 1, 2, 3, 4, 5, 6, 8, 9 and 10 rotaviruses. There was, however, significant neutralization by rabbit serum against avian rotavirus PO-13 (Table 4). Three guinea pig hyperimmune sera were raised against isolate 993/83. Significant neutralization of serotype 3 and serotype 7 rotaviruses was observed with all three sera. Reassortant rotavirus N-4, containing SA11-derived VP7 and NCDV-derived VP4, was significantly neutralized by all three sera, whereas reassortant rotavirus S-4, containing SA11-derived VP4 and NCDV-derived VP7, was not significantly neutralized (Table 5). Rhesus rotavirus (RRV) was also neutralized with high titers. RRV reassortants in which RRV-specific VP7 was replaced by serotype 2- or serotype 4-specific VP7 were not neutralized (Table 5). Notably, rabbit serum against avian rotavirus PO-13 also neutralized serotype 3 simian rotavirus SA11 at a high titer (Table 4).

RNA-RNA hybridization. When the ^{32}P-labeled transcription probe prepared from the 993/83 strain was hybridized with the genomic RNA from pigeon rotavirus strain PO-13, eight hybrid bands that did not comigrate with the genomic RNA segments of PO-13 were observed (Fig. 1). Correspondingly, the probe prepared from the PO-13 strain produced nine bands with the genomic RNA from the 993/83 strain. Formation of hybrids that did not comigrate with genomic RNA segments was observed. Such hybrids often appeared as extra bands on ethidium bromide-stained gels,

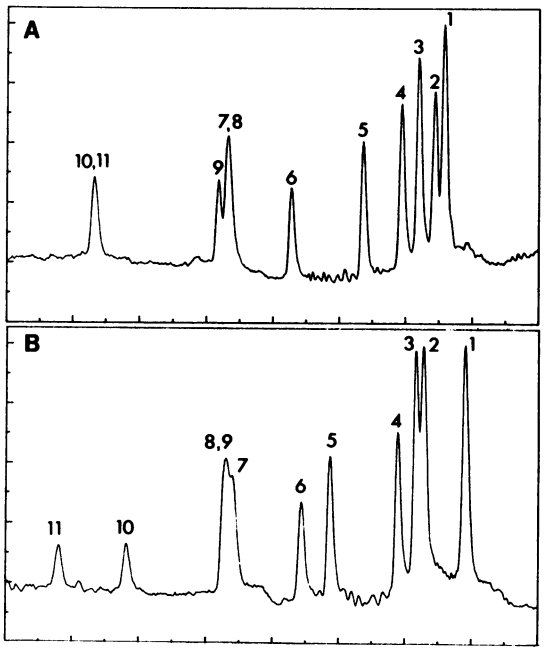

FIG. 2. Densitometric tracings of the genome profiles of isolate 993/83 (A) and bovine rotavirus isolate 675/78 (B) (described in reference 3) are shown. Peak numbers correspond to gene segment numbers.

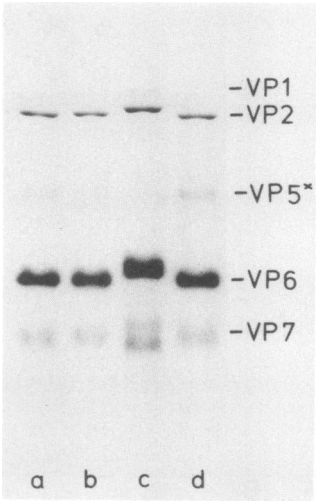

FIG. 4. SDS-PAGE of Coomassie blue-stained structural polypeptides of double-shelled virions of bovine rotavirus isolates 125/83 (lane a, serotype 6), 1446/78 (lane b, serotype 6), 993/83 (lane c), and 522/79 (lane d, serotype 6), described in reference 3. $VP5^x$ is the large tryptic cleavage fragment of VP4 encoded by gene segment 4.

whereas homologous RNA-RNA reactions were always identified by bands on the autoradiograms that comigrated with genomic double-stranded RNA segments visualized by ethidium bromide staining of the gel. We interpret those hybrid bands which did not comigrate with the genomic RNA segments as representing hybrid molecules that were different in their secondary structures from the homologous genomic RNA molecules; hence, they had different mobilities on gel electrophoresis. Although aberrant migration of the hybrid bands suggested some sequence heterogeneity of genomic RNA between these two strains, this level of

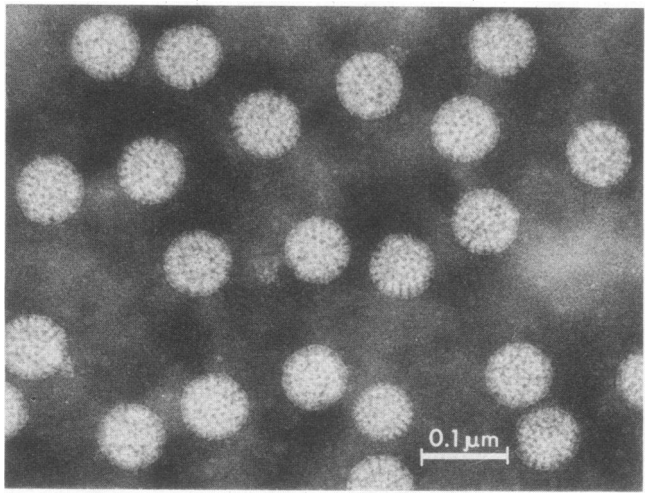

FIG. 3. Electron micrograph of extracellular rotavirus 993/83 purified by CsCl density gradient centrifugation and negatively stained with 2% phosphotungstic acid.

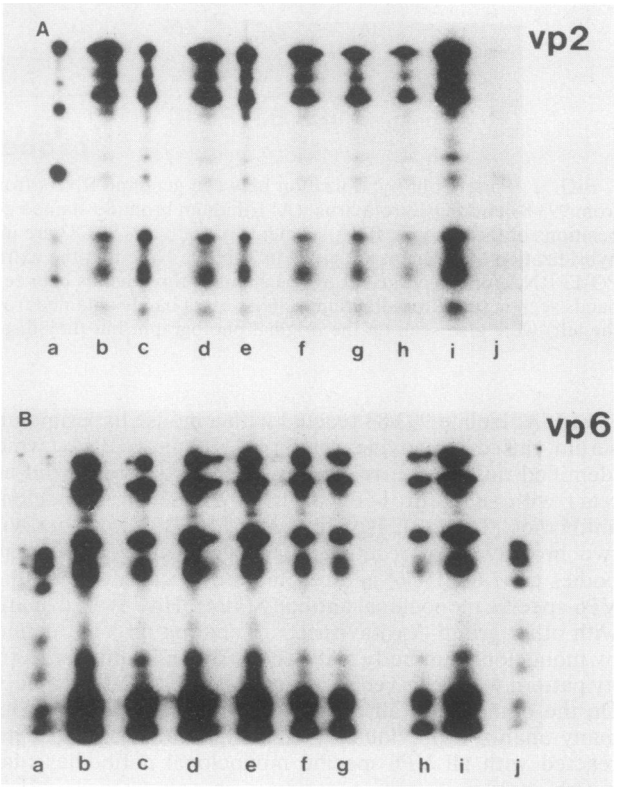

FIG. 5. Limited-proteolysis analyses of VP2 (A) and VP6 (B) of isolate 993/83 (lanes a and j) are shown, in comparison with bovine rotaviruses NCDV (lanes b), 1446/78 (lanes c, serotype 6), 667/77 (lanes d, serotype 6), 10/78 (lanes e, serotype 10), V1005 (lanes f, serotype 10), 709/83 (lanes g, serotype 6), 479/80 (lanes h, serotype 10), and 125/83 (lanes i, serotype 6). [^{35}S]methionine-labeled polypeptides were digested with *S. aureus* V8 protease.

TABLE 3. Typing of rotavirus isolate 993/83 with monoclonal antibodies to VP6 by ELISA

Antibody	Specificity	ELISA OD_{404} with indicated rotavirus antigen[a]				
		NCDV	SA11	KU	993/83	PO-13
Mouse serum	Polyclonal	0.57	0.81	0.69	0.66	0.60
256/60	Subgroup I[b]	0.36	0.46	0.02	0.05	0.06
631/9	Subgroup II[b]	0.02	0.01	0.32	0.01	0.06
5D7	VP6[c]	0.45	0.76	0.63	0.01	0.08
4B4	VP6[c]	0.27	0.32	0.30	0.09	0.01
5A8	VP6[c]	0.34	0.45	0.50	0.95	0.30
5C6	VP6[c]	0.35	0.39	0.23	0.03	0.07

[a] Optical density at 404 nm (OD_{404}) was <0.02 for control plates with coating buffer only.
[b] Specificity from the work of Greenberg et al. (14).
[c] Specificity from the work of Gerna et al. (12).

homology was considered high enough for isolate 993/83 and PO-13 to be grouped together on the basis of their genetic homology, which we termed genogroup (29). It was previously shown that rotaviruses recovered from the same animal species had a high degree of homology with each other but that this level of homology was not detected between the viruses recovered from different animal species (10). To examine whether 993/83 showed any degree of homology with other mammalian rotavirus strains, denatured genomic RNAs of 12 rotavirus strains derived from seven different mammalian host species were allowed to hybridize with the 993/83 probe (Fig. 1). None of the genomic RNA segments from these rotaviruses of mammalian host origin showed homology with the 993/83 probe, indicating that the genetic constitution of the 993/83 strain was quite distinct from that of mammalian rotavirus strains.

DISCUSSION

Four lines of evidence indicate that rotavirus 993/83, isolated from a symptomatic calf, might be not of bovine but of avian origin. Isolate 993/83 showed extensive RNA homology with a pigeon rotavirus isolate but no detectable RNA homology with mammalian rotaviruses isolated from eight different species, including two serotypes of bovine rotaviruses. Previously, it had been shown that rotavirus strains derived from the same animal species (such as cattle, pigs, and dogs) have a high degree of RNA homology (10, 21, 25, 32). In addition, the electropherotype of isolate 993/83 is highly unusual for bovine rotaviruses but very similar to RNA profiles of avian rotaviruses (38, 39). A third line of evidence for the avian origin of isolate 993/83 is the absence of subgroup I and II specificities on VP6 of this isolate. All avian rotaviruses from America and Northern Ireland examined so far (14, 38) lacked both subgroup antigens, whereas all known bovine rotavirus isolates showed subgroup I reactivity. Isolate 993/83 and avian rotavirus PO-13 also shared the property of reacting with only one of four broadly cross-reacting VP6-specific monoclonal antibodies. Peptide mapping confirmed that VP6 and VP2 of isolate 993/83 clearly differed from the corresponding polypeptides of a panel of bovine rotavirus isolates. This observation is significant in view of the conservative behavior of VP6 revealed for mammalian rotaviruses by peptide mapping (7) and direct gene sequencing (13).

Finally, the cross-neutralization test revealed a two-way cross-reaction between isolate 993/83 and avian rotavirus PO-13. Until now, serotype 7 reactivity has been restricted to avian rotaviruses (9). The one-way cross-reaction between isolate 993/83 and two serotype 3 simian rotaviruses is surprising, as no RNA homology was detected.

There is some evidence that the sharing of genogroup specificity indicated interspecies transmission of rotaviruses (12a, 31, 33). Isolate 993/83 could thus represent a candidate

TABLE 5. Reactivity of 993/83-specific guinea pig hyperimmune sera with serotype 3 simian rotaviruses

993/83 antiserum	Titer of neutralizing antibody to indicated rotavirus strain					
	SA11	S-4	N-4	RRV	DS-1 × RRV	ST3 × RRV
A	6,400	100	800	3,200	<100	<100
B	3,200	<100	3,200	1,600	<100	<100
C	12,800	400	12,800	25,800	<100	<100

TABLE 4. Serological characterization of rotavirus isolate 993/83 by animal hyperimmune sera raised against 10 rotavirus serotypes

Antiserum specificity strain (serotype)	Titer of neutralizing antibody to indicated rotavirus strain (serotype)											
	Wa (1)	S-2 (2)	SA11 (3)	Hochi (4)	OSU (5)	NCDV (6)	PO-13 (7)	678 (8)	WI61 (9)	B223 (10)	V1005 (10)	993/83
Wa (1)[a]	12,000						<100					<50
S-2 (2)[a]		1,600					<100					<50
SA11 (3)[a]			12,000				<100					<50
Hochi (4)[a]				12,000			<100					<50
OSU (5)[b]					200,000		100					200
NCDV (6)[a]						12,000	<100					<100
PO-13 (7)[c]			3,200				6,400					3,200
678 (8)[a]							<100	12,000				<50
WI61 (9)[b]							<200		6,400			200
B223 (10)[a]							<100			6,400		<100
V1005 (10)[a]							<100				6,400	<50
993/83 (A)[d]	<100	<100	6,400	<100	<100	100	800	200	<100	100	<100	3,200
993/83 (B)	<100	<100	3,200	<100	<100	<100	12,000	<100	<100	100	<100	8,000
993/83 (C)	<100	<100	12,800	<100	<100	<100	24,000	<100	<100	400	<100	12,800

[a] Guinea pig hyperimmune serum characterized previously (4).
[b] Rabbit hyperimmune serum characterized previously (4).
[c] Guinea pig hyperimmune serum characterized previously (21).
[d] A, B, and C identify the three antisera (see Table 5).

for an interspecies transmission of a rotavirus between different classes of vertebrates. In view of the possible virological significance of this finding, we should stress that neither avian rotaviruses nor bird feces were ever introduced into the Munich laboratory. Avian rotaviruses were introduced into the Lausanne laboratory only in the final phase of the characterization of the 993/83 isolate. Laboratory contamination thus seems a very remote possibility. In addition, the four nearly equidistant group I RNA segments (34), the widely spaced group II RNA segments, and the comigrating group IV RNA segments were already observed in the original stool sample (8). The quality of the original documentation did not permit a comparison of the similarity of the group III RNA genes. The original stool sample was unfortunately exhausted in the cell culture adaptation assays. Thus, we could not prove that the RNA segments of the cell culture-adapted rotavirus comigrated with the original stool rotavirus. A field contamination also seems a very improbable explanation. Stool samples from diarrheic calves were taken directly from the animal and were not recovered from the ground. In addition, we have sought and identified both the clinical chart of the calf whose feces gave rise to the 993/83 isolate and the name of the local veterinarian. Erroneous exchange with an avian stool sample in the diagnostic center is improbable, as no avian microbiological analysis was performed at the diagnostic center. Infection of calves with a putative avian rotavirus is, in any case, a rare event. During the years 1978 to 1985, 1,430 fecal samples were obtained from German calves with diarrhea. Only four samples showed an unusual pattern of genomic RNA for group A bovine rotaviruses (8). Three stool samples showed a pattern similar to that described for group B rotaviruses (36). Isolate 993/83 was thus 1 of 1,430 isolates tested whose electropherotype indicated an avian origin.

Further studies are necessary to substantiate our suspicion of an interspecies transmission of rotaviruses between birds and mammals. Comparative analyses of isolate 993/83 and a panel of avian rotaviruses are needed to assess whether isolate 993/83 is a typical avian rotavirus or whether it shares only some of the properties of avian rotaviruses. Analyses might also reveal which avian rotaviruses are the closest relatives of isolate 993/83. One should also try to identify the characteristic electropherotype of the 993/83 isolate with stool samples of symptomatic birds in Germany. A visit to several German dairy farms revealed sparrows, swallows (summer only), pigeons, crows, and owls (winter only) in cattle sheds. Lack of the original stool sample, however, will hamper efforts to evaluate the virulence of the 993/83 isolate for different species of birds and mammals.

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