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Characterization of the Bovine Group A Rotavirus Strain Neonatal Calf Diarrhea Virus-Cody (NCDV-Cody)†

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The neonatal calf diarrhea virus-Cody (NCDV-Cody) strain was found to contain a mixture of rotaviruses with G6 and G8 VP7 genes. Challenge exposure of calves with the mixed virus inoculum indicated that both viruses were maintained by passage in vivo. This is the first P1:G8 rotavirus to be characterized in cattle in the United States.

Rotaviruses (RVs) are members of the family *Reoviridae* and contain 11 distinct gene segments of double-stranded RNA enclosed in a double-shelled protein capsid (5). Seven different groups of RVs have been identified, with group A RVs being the most common cause of neonatal diarrhea in animals and human beings (25). The outer capsid of RVs consists of two viral proteins—VP4 or P, for protease sensitivity, and VP7 or G, for glycoprotein—which contain epitopes independently involved in the neutralization of virus infectivity in vivo and in vitro (11, 21). Group A RVs have been assigned to different serotypes on the basis of different VP4 and VP7 proteins and genes (5). Fourteen distinct VP7 genes have been reported among group A RVs (3, 5, 12), with eight different genotypes—namely, G1, G2, G3, G6, G8, G10, G11, and untypeable—occurring in cattle (1, 13, 15, 22, 27).

Bovine RV (BRV) strain neonatal calf diarrhea virus-Lincoln (NCDV-Lincoln) is the reference strain for group A RV G serotype 6 (5, 12). The NCDV-Cody strain of BRV is considered the virulent counterpart for the attenuated NCDV-Lincoln strain present in commercially available vaccines for cattle. These strains were obtained by Mebus and coworkers (16, 19) from outbreaks of neonatal calf diarrhea concurrently occurring in Nebraska. Strain NCDV-Lincoln was isolated from a diarrheic calf on a dairy farm, located in Lincoln County, Nebr., while the NCDV-Cody strain was obtained from a diarrheic beef calf on a beef cow-calf ranch near the town of Cody, Nebr. (16, 19). The fecal material collected during the Cody outbreak subsequently was serially passaged by oral inoculation of calves interspersed with occasional passages in cell cultures (Table 1) and was designated as either the Cody isolate of the NCDV agent (6, 32, 33) or NCDV-Cody (18, 22, 26, 28). Intestinal contents from calf GN79-11 and calf GN81-4 were distributed to research laboratories for use either as reference material in comparative studies (22, 23) or as inoculum in physicochemical characterizations (31–33) or calf challenge experiments (17, 18, 26, 28–30). Although some information about the P and G compositions of a cell culture-adapted isolate derived from the intestinal contents of a calf-passaged NCDV-Cody is available (22, 23), discrepancies with the results obtained in our laboratories as well as others (34) prompted us to examine the NCDV-Cody material in more detail.

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The first passage of GN69-2 in fetal bovine lung cells, designated CC69-2, was obtained from C. A. Mebus's original collection (Table 1). Isolate CC69-2 was plaque purified three times in rhesus monkey kidney (MA-104) cells in our laboratory as described previously (12) and was designated PP69-2. Similarly, intestinal contents from calf GN81-4 were obtained from A. Torres's collection of research materials and were adapted to MA-104 cells in our laboratory, and an isolate plaque purified three times was designated PP81-4. Reference strains for each G serotype included Wa (G1), DS-1 (G2), P (G3), ST3 (G4), and OSU (G5), obtained from E. H. Bohl, Ohio Agricultural Research and Development Center, Ohio State University; NCDV-Lincoln (G6), obtained from the American Type Culture Collection, Rockville, Md.; B641 (G6), obtained from G. N. Woode, College of Veterinary Medicine, Texas A&M University, College Station; Ch2 (G7) and 69M (G8), obtained from S. Matsuno, Central Virus Diagnostic Laboratory, National Institute of Health, Tokyo, Japan; 678 (G8), obtained from D. R. Snodgrass, Animal Disease Research Association, Moredun Institute, Edinburgh, Scotland; WI61 (G9), obtained from H. F. Clark, The Wistar Institute of Anatomy and Biology, Philadelphia, Pa.; B223 (G10), obtained from G. N. Woode; YM (G11), obtained from C. F. Arias, Centro de Investigaciones Sobre Ingenieria Genetica y Biotecnologia, Cuernavaca, Mexico; L26 (G12), obtained from S. Urasawa, Sapporo Medical College, Sapporo, Japan; and L338 (G13) and FI23 (G14), obtained from D. R. Snodgrass. Additionally, single-gene reassortants between strains UK and 69M and strains DS-1 and 678 that contained the G8 VP7 gene were prepared as described previously (10). All reference viruses were grown in MA-104 cells as described previously (14).

Plaque reduction neutralization assays (12) with guinea pig serum hyperimmune to reference G serotypes 1 through 14 and isolates PP69-2 and PP81-4 indicated a strong antigenic relationship between isolates PP69-2 and PP81-4, serotype G8 strains 69M and 678, and single-gene reassortants UK × 69M and DS-1 × 678 containing the G8 VP7 gene (Table 2). Two neutralizing monoclonal antibodies (N-MAbs), designated 20/12 and 110/63, specific for isolate PP81-4 were prepared by

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TABLE 1. Passage history of group A BRV strain NCDV-Cody in calves and cell culture

Passage	Reference
Fecal material ↓ CD[a]68-1 ↓ GN[b]68-2 ↓ GN68-7 ↓ CD68-8 ↓ CD68-10 ↓ GN68-22 ↓	Mebus et al., 1969
GN68-42 → EBT[c]-1 → EBT-2 ↓ CD68-49 → FBL[d]-1 → FBL-2 → FBL-3 ↓ GN69-2 → FBL (CC[e]69-2) ↓	Mebus et al., 1971
GN70-9 ↓ GN71-20 ↓ GN71-61 ↓ GN72-48 ↓ GN73-12 ↓ GN73-38 ↓ GN74-116 ↓ GN74-163 ↓ GN78-15 ↓ GN79-3 ↓ GN79-11 ↓ GN81-4 ↓ ↓	A. Torres[f]
GN88-1 GN90-1	This report

[a] CD, colostrum-deprived calf.
[b] GN, gnotobiotic calf.
[c] EBT, embryonic bovine tracheal cells.
[d] FBL, fetal bovine lung cells.
[e] CC, cell culture adapted.
[f] Unpublished data generated at the University of Nebraska-Lincoln between 1970 and 1981.

a previously described method (14). Ascitic fluids containing PP81-4-specific N-MAbs were tested against reference RV G serotypes 1 through 14, including BRVs B641 and 678 and the single-gene reassortants UK × 69M and DS-1 × 678, by plaque reduction or fluorescent focus neutralization assays (14). The N-MAbs had antibody titers greater than 20,000 with isolate PP81-4 but less than 80 with all other 14 reference G serotypes, strains 69M and 678, and the single-gene reassor-

TABLE 2. Antigenic relationships between BRV isolates PP69-2 and PP81-4 and reference group A RV G serotypes 1 through 14 as determined by plaque reduction assay

Virus	VP7	Reciprocal of 60% plaque reduction antibody titer of hyperimmune guinea pig serum to the indicated rotavirus[a]																			
		Wa	DS-1	P	ST3	OSU	UK	NCDV-Lincoln	Ch2	69M	678	PP81-4	PP69-2	WI61	B223	YM	L26	L338	FI23	UK × 69M	DS-1 × 678
Wa	1	**40,960**										160	<80								
DS-1	2		**10,240**									80	<80								
P	3			**20,480**								640	640								
ST3	4				**10,240**							80	<80								
OSU	5					**40,960**						2,560	<80								
UK	6						**40,960**					<80	<80								
NCDV-Lincoln	6							**20,480**				2,560	<80								
Ch2	7								**10,240**			<80	<80								
69M	8									**10,240**		10,240	2,560								
678	8										**20,480**	10,240	ND								**20,480**
PP81-4		160	80	640	<80	160	160	640	<80	10,240	40,960	**10,240**	ND	<80	320	160	<80	640	<80	10,240	10,240
PP69-2		640	640	1,280	160	640	2,560	2,560	160	40,960	10,240	40,960	**10,240**	160	640	640	160	2,560	160	40,960	40,960
WI61	9											<80	<80	**20,480**							
B223	10											<80	<80		**10,240**						
YM	11											640	640			**20,480**					
L26	12											80	<80				**10,240**				
L338	13											160	640					**20,480**			
FI23	14											80	<80						**20,480**		
UK × 69M	8											10,240	ND							**20,480**	
DS-1 × 678	8											10,240	ND								**20,480**

[a] Values indicating homology are in boldface type. ND, not determined.

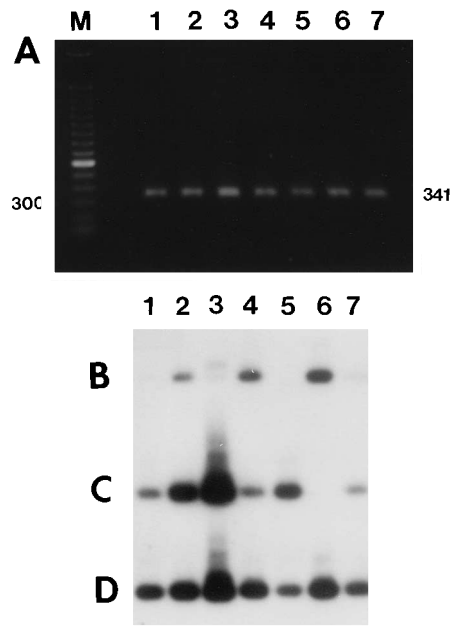

FIG. 1. Southern blot hybridization of RT-PCR-generated G-specific cDNA products from cell culture-adapted and calf-passaged NCDV-Cody strain with G-specific cDNA probes. (A) cDNA-amplified products of 341 kb electrophoresed on a 1% agarose gel and stained with ethidium bromide. Transferred cDNA from the gel in panel A hybridized with RT-PCR-generated [α-^{32}P]dCTP-labeled G-specific cDNA of RNA extracted from strain NCDV-Lincoln (B), isolate PP81-4 (C), and the intestinal contents of calf GN81-4 (D). Lanes: M, molecular size standard (1-kb DNA ladder; GIBCO-BRL); 1, 69M; 2, CC69-2; 3, PP69-2; 4, GN81-4; 5, PP81-4; 6, NCDV-Lincoln; 7, 678.

tants. N-MAb 20/12 had a neutralizing antibody titer greater than 20,000 with isolate PP69-2. These results suggested the existence of strain-specific epitopes on VP7 protein encoded by the G8 genes of PP69-2 and PP81-4.

To confirm the presence of a G8 VP7 gene in biologically cloned derivatives and uncloned NCDV-Cody, two primers complementary to nucleotides 51 to 71 of the minus strand (5′-GTATGGTATTGAATATACCAC-3′) and nucleotides 376 to 392 of the plus strand (5′-GATCCTGTTGGCCATCC-3′) of the group A RV G VP7 gene (8) were synthesized (National Sciences, Plymouth, Minn.). Viral RNA extracted from cell cultures inoculated with NCDV-Lincoln, 69M, 678, CC69-2, PP69-2, or PP81-4 and intestinal contents from calf GN81-4 were denatured at 80°C for 5 min and reverse transcribed with reverse transcriptase (RT), and the 341-kb cDNA fragments (Fig. 1A) were amplified by PCR as described previously (14). After separation by electrophoresis in 1% agarose gels, the amplified cDNA products were transferred to nylon membranes by Southern blotting, and membranes were hybridized with RT-PCR-generated [α-^{32}P]dCTP-labeled G-specific cDNA probes of viral RNA from strain NCDV-Lincoln and isolate PP81-4 by using an oligolabeling kit (Pharmacia, LKB Biotechnology, Piscataway, N.J.). Prehybridization and hybridization were done in a solution containing 1% sodium dodecyl sulfate (SDS); 0.25% each of bovine serum albumin, polyvinylpyrrolidone, and Ficoll; and 5× SSPE (1× SSPE contains 0.15 M NaCl, 0.01 M NaH_2PO_4, 0.001 M EDTA [pH 7.4]), each for 1 h at 62°C. The membranes then were washed in 1× SSPE with 1% SDS (twice for 5 min each time at room temperature and once for 5 min at 62°C). Exposure of the membranes to X-Omat AR Cronex radiograph film (Eastman Kodak Company, Rochester, N.Y.) in a cassette with Lightning Plus intensifying screens (DuPont, Wilmington, Del.) at −70°C

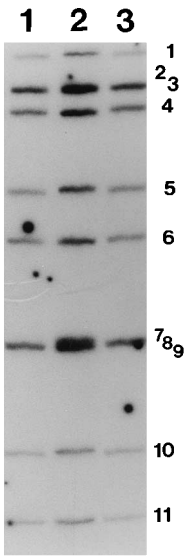

FIG. 2. Northern blot hybridization of cell culture-adapted and plaque-purified NCDV-Cody G8 isolates with a whole genomic RNA probe prepared from RNA of strain NCDV-Lincoln. The gene segments are numbered on the right according to the order of migration. Lanes: 1, PP81-4; 2, NCDV-Lincoln; 3, PP69-2.

revealed a positive hybridization signal between the G-specific cDNA probe from isolate PP81-4 and isolate PP69-2 and strains 69M and 678 but not with strain NCDV-Lincoln, indicating the presence of a G8 VP7 gene in the PP69-2 and PP81-4 isolates (Fig. 1B). Conversely, the G-specific cDNA probe from strain NCDV-Lincoln did not react with isolates PP69-2 and PP81-4 or strains 69M and 678 by Southern blotting, indicating that these isolates did not contain a G6 VP7 gene (Fig. 1B). Hybridization of probes from strain NCDV-Lincoln and isolate PP81-4 with G-specific cDNA from CC69-2 and GN81-4 confirmed that BRVs with G6 and G8 VP7 genes were maintained by passage in vitro and in vivo, respectively (Fig. 1B).

Other gene segments of isolates PP69-2 and PP81-4 were characterized further by Northern blot hybridization. Viral RNAs of strain NCDV-Lincoln and isolates PP69-2 and PP81-4 were obtained by phenol-chloroform extraction of infected cell cultures, separated by electrophoresis on an SDS–10% polyacrylamide gel, and transferred to a nylon membrane as described previously (9). Hybridization of membrane-bound RNA with an [α-^{32}P]GTP-labeled whole genomic RNA probe of strain NCDV-Lincoln prepared by in vitro transcription (9) revealed that these BRVs were homologous at all gene segments, including the P1 gene (Fig. 2). Minor differences in migration patterns of gene segments 1, 5, and 10 of isolates PP69-2 and PP81-4 by SDS-polyacrylamide gel electrophoresis (PAGE) suggested gene reassortment during passages either in vivo or in vitro of the uncloned strain NCDV-Cody.

To confirm that BRVs with G6 and G8 VP7 genes could be maintained by passage in vivo, intestinal contents from calf GN81-4 was inoculated into susceptible calves. Two gnotobiotic calves, designated GN88-1 and GN90-1 (Table 1) (20), were orally inoculated at approximately 3 h of age with 3 ml of intestinal contents from calf GN81-4 mixed with 7 ml of sterile Eagle's minimal essential medium (MEM; Gibco Laboratories, Grand Island, N.Y.). At approximately 15 h postinoculation, the calves were euthanized and the intestinal contents were harvested. Fifteen calves were caught at birth in sterile towels, placed in individual sterile isolators (20), and fed 1 liter of a

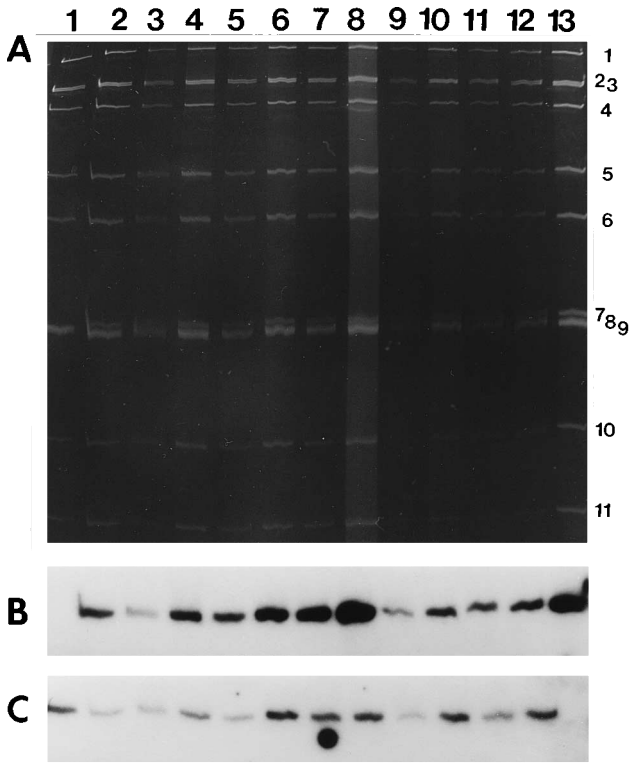

FIG. 3. Northern blot hybridization of RNA extracted from the intestinal contents of gnotobiotic calves and fecal samples obtained postinoculation of conventional calves with GN81-4 materials with G-specific cDNA probes. (A) RNA from each intestinal sample was electrophoresed on an SDS–10% polyacrylamide gel, and the gel was stained with ethidium bromide. The gene segments are numbered on the right according to the order of migration. The transferred RNA from the gel in panel A was hybridized with RT-PCR-generated [α-^{32}P]dCTP-labeled G-specific cDNA probes of NCDV-Lincoln (B) and PP81-4 (C) generated by RT-PCR. Lanes: 1, PP81-4; 2, GN88-1; 3, C1-1; 4, C1-2; 5, C1-3; 6, C1-4; 7, C1-5; 8, GN90-1; 9, C2-1; 10, C2-2; 11, C2-3; 12, C2-4; 13, NCDV-Lincoln.

1:10 dilution of pooled normal colostrum in phosphate-buffered saline within 4 h of birth. Oral inoculation of these calves 6 h later with 3 ml of intestinal contents from either calf GN88-1 (calf 1-1 [C1-1] to C1-6) or calf GN90-1 (C2-1 to C2-9) mixed with 7 ml of MEM resulted in severe watery diarrhea within 33.3 $\pm$ 10.8 h in all of the calves. Twenty percent of the calves died or had to be euthanized because of severe dehydration associated with profuse diarrhea. Fecal samples collected daily were positive for the presence of RV as determined by negative staining with phosphotungstic acid (2) and transmission electron microscopy and by SDS-PAGE analysis of purified RNA (14). Positive hybridization signals between viral RNAs obtained from the intestinal contents of calves GN88-1 and GN90-1 and from fecal samples collected at the onset of diarrhea from nine conventional calves, when they were reacted with [α-^{32}P]dCTP-labeled G-specific cDNA probes of isolate PP81-4 and strain NCDV-Lincoln by Northern blotting (9) (Fig. 3), confirmed that BRVs with G6 and G8 VP7 genes were maintained by passage in vivo. These results support previous reports suggesting mixed BRV infection as a cause of naturally acquired neonatal calf diarrhea (4, 7, 24).

Considering the origins of the materials used for the propagation of NCDV-Lincoln and NCDV-Cody and the diversity of BRV genotypes that occur in the United States, the identification of BRVs with different VP7 genes in NCDV-Lincoln and NCDV-Cody was not surprising. However, the identification of BRVs with G6 and G8 VP7 genes in uncloned NCDV-

Cody samples separated by 12 passages from 1969 to 1981 (isolates PP69-2 and PP81-4; Table 1) was not expected and might explain some of the discrepancies observed between laboratories. It is possible that these viruses were present in the original NCDV-Cody material; however, in view of the passage history of NCDV-Cody, contamination with an RV containing a VP7 gene different from that in the virus in the original field sample cannot be ruled out entirely. Results of Northern blot analyses of the calf-passaged GN81-4 material (Fig. 3) support the hypothesis that BRVs with G6 and G8 VP7 genes were maintained by passage in vivo.

Limited epidemiological data on the prevalence of BRV G types in feces obtained from calves with neonatal diarrhea indicate that the G6 VP7 gene is the most prevalent (22, 27). Only 2.9% of 102 RNA samples obtained from the feces of calves with neonatal diarrhea examined by Parwani and coworkers (22) were positive for the presence of the G8 VP7 gene, which was determined by using partial-length cDNA probes. None of the G8 BRVs identified in that study were isolated or characterized. The PP69-2 and PP81-4 isolates thus represent the first biologically cloned and characterized serotype G8 BRVs identified in cattle in the United States. The presence of a BRV with a G8 VP7 gene in material collected by Mebus and coworkers (19) suggests that this serotype was present in beef cattle in the United States more than two decades ago.

Results from the present study raise important questions about the interpretation of results obtained in previous studies in which uncloned NCDV-Cody material was used. Biological cloning of different viruses in different laboratories may account for some of the discrepancies observed. However, of greater concern is the importance of determining the composition of reference material for future clinical and laboratory studies aimed at assessing the efficacies of vaccines or antisera for the prevention of neonatal calf diarrhea caused by BRVs.

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