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Detection of Rotavirus Serotypes G1, G2, G3, and G11 in Feces of Diarrheic Calves by Using Polymerase Chain Reaction-Derived cDNA Probes†

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On the basis of antigenic variability in the VP7 outer capsid glycoprotein, at least 14 G serotypes exist for group A rotaviruses. Serotypic diversity exists among bovine rotaviruses (BRV), with serotypes G1, G6, G8, and G10 reported for cattle. Although G1 and G8 rotaviruses were originally described for humans, the recent isolation of G6 and G10 rotaviruses from humans further emphasizes the serotypic similarity between human and bovine rotaviruses and the possible zoonotic potential of rotaviruses. Results of our previous studies have indicated that more than 24% of BRV-positive field samples from diarrheic calves were nonreactive with cDNA probes or monoclonal antibodies to serotypes G6, G8, and G10. In this study, cDNA probes were prepared by polymerase chain reaction amplification of the hyperdivergent regions of the VP7 genes (nucleotides 51 to 392) from human (G1, G2, and G3) and porcine (G4, G5, and G11) rotaviruses. These probes were used in a dot blot hybridization assay to further characterize the G types of 59 BRV strains (fecal samples from diarrheic calves in Ohio, Nebraska, Washington, and South Dakota) that were nonreactive with cDNA probes to G6, G8, and G10. Rotaviruses belonging to serotypes G1 (n = 7), G2 (n = 1), G3 (n = 2), and G11 (n = 3) were identified among the BRV field samples. The BRV associated with these G types accounted for 22% of the samples tested; the other 78% of these samples remained untypeable with these probes. To our knowledge, this is the first report in the United States of the identification among BRV isolates of rotavirus serotypes G1, G2, G3, and G11.

Group A rotaviruses are a major cause of diarrhea in the young of many species, including humans, throughout the world (4, 38, 42, 47, 51). The viral genome consists of 11 segments of double-stranded RNA (dsRNA) surrounded by a double capsid (15, 28). The outer capsid shell consists of a 37-kDa glycoprotein, VP7, encoded by gene segment 7, 8, or 9, depending on the virus strain, and VP4, with a molecular mass of 86 kDa, encoded by gene segment 4 (15, 28, 42). VP4 and VP7 elicit production of neutralizing antibodies (15, 25–27, 34, 46, 53). Analysis of reassortant viruses has also shown that serotype-specific neutralization epitopes reside on VP7 (15, 25–27, 34, 46). Classification of group A rotavirus serotypes has been primarily based on the specificity of the VP7 antigen (G serotypes) (15, 16, 21, 32) and to a limited extent on the VP4 antigen (P serotypes) (15, 32, 45). A total of 14 G serotypes have been described to date (9, 10, 13, 14, 22, 23, 26, 37, 48, 52, 54). Serotypes G6 and G10 have been reported to be the most common types found in cattle (4, 14, 49). However, Snodgrass et al. (44) reported bovine rotavirus (BRV) isolates of serotype G8. Recently, rotavirus serotype G1, which is typically associated with infections in humans, was found in bovine (6) and porcine (5) populations. Furthermore, it was reported that some rotaviruses detected in cattle were antigenically related to serotypes G1, G2, and G3, as determined by using monoclonal antibodies to the respective serotypes of human rotavirus in enzyme-linked immunosorbent assays (ELISA) (7). Although neutralizing antibodies against animal rotavirus serotypes G5, G6, G7, and G10 have been found in children, rotaviruses of these G types were not isolated from the feces of the seropositive children (12). However, recent studies have identified at least two human rotaviruses with G6 specificities (19) and one human rotavirus with G10 specificity (3), serotypes previously recognized only in cattle.

Our objective was to further characterize 59 BRV strains that were nonreactive with G6, G8, and G10 cDNA probes (37). These 59 samples were tested with G-type cDNA probes of human (G1, G2, and G3) and porcine (G4, G5, and G11) origin. Fecal samples were obtained from calves (1 to 30 days old) with diarrhea and were from Ohio (n = 35), Nebraska (n = 9), South Dakota (n = 12), and Washington (n = 3). The field specimens were classified as positive or negative for group A rotavirus on the basis of one or more of the following assays: cell culture immunofluorescence (50), immune electron microscopy (8), ELISA (29), or polyacrylamide gel electrophoresis (PAGE) (24). Two fecal specimens obtained from uninoculated gnotobiotic calves and confirmed as negative by the tests listed above were used as negative controls. Positive controls included the BRV strains NCDV (Nebraska calf diarrhea virus) (G6P1), IND (G6P5), and Cr (G10P11), which were described previously (36).

Extraction of rotavirus dsRNA from fecal samples was performed by methods similar to those described previously (40). Briefly, samples were diluted 1:5 in Tris-$CaCl_2$ buffer (0.1 M Tris, 10 mM $CaCl_2$ [pH 7.4]). The diluted samples were vortexed and clarified by centrifugation at 1,200 × g for 30 min at 4°C. Supernatants were collected, and sodium dodecyl sulfate (SDS) and sodium acetate were added to concentrations of 1.0% and 0.1 M, respectively. The suspension was extracted with an equal volume of phenol-chloroform (1:1), and then 4 M sodium acetate (0.1 volume) and

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100% ethanol were added to the aqueous phase. The RNA was precipitated overnight at −20°C, resuspended in diethylpyrocarbonate-treated water (500 to 800 μl), and stored at −20°C. The concentrations of nucleic acid in samples were estimated by determining the A_{260} reading in a spectrophotometer. The estimated concentrations represented the total amount of nucleic acid present in the extracted samples and did not reflect rotavirus-specific dsRNA only (41).

Rotavirus dsRNA in extracted samples was confirmed by PAGE. Electrophoresis was performed with the discontinuous buffer system of Laemmli (30). Rotavirus dsRNA was resolved in 10% polyacrylamide slab gels, and electrophoresis was conducted at 12 mA for 14 to 16 h. The RNA bands were visualized by the silver staining method of Herring et al. (24).

The full-length cDNA copies of the VP7 gene from the BRV strains NCDV (G6) and Cr (G10) inserted into pGEM and pBS plasmids were prepared in our laboratory (36). Full-length cDNA copies of the VP7 gene from human rotavirus isolates Wa (G1), DS-1 (G2), P (G3), and 69M (G8) inserted into pUC13 plasmids were kindly provided by J. Flores (National Institute of Allergy and Infectious Diseases, Bethesda, Md.). Full-length cDNA copies of gene 9 (VP7 gene) from Gottfried (G4) and OSU (G5) porcine rotaviruses inserted in the plasmid vectors pBR322 and pTZ18R, respectively, were provided by M. Gorziglia (National Institute of Allergy and Infectious Diseases). The YM gene 9 cDNA of porcine rotavirus (G11) inserted in the pGEM plasmid was provided by Susan López (2) (Division of Biology, California Institute of Technology, Pasadena, Calif.). All of the full-length cDNA clones of the VP7 genes were used as templates for polymerase chain reaction (PCR) amplification by methods described previously (17, 18, 36).

For PCR amplification of short cDNA segments (341 nucleotides), primers A (5′-GTATGGTATTGAATATAC CAC-3′, homologous to nucleotides 51 to 71 of the VP7 gene) and B (5′-GATCCTGTTGGCCATCC-3′, complementary to nucleotides 376 to 392 of the VP7 gene) were used. These primers corresponded to highly conserved regions of the VP7 gene and flanked the target G serotype-specific regions. The PCR mixture consisted of 200 μM (each) dATP, dCTP, dGTP, and dTTP; 20 mM Tris-HCl, pH 8.3; 2.5 mM $MgCl_2$; 0.05% gelatin; 200 ng of each primer; 1 to 10 ng of DNA template; and 2.5 U of *Taq* polymerase (Boehringer Mannheim Biochemicals, Indianapolis, Ind.). Thirty amplification cycles were used, each consisting of 94°C for 1 min (denaturation), 42°C for 1.5 min (annealing), and 72°C for 2.5 min (extension). The PCR products were purified by Centiflex-AG cartridges (Advanced Genetic Technologies Corp., Gaithersburg, Md.) and analyzed by electrophoresis on 1% agarose gels by standard procedures (43). The sizes of the PCR products were confirmed by visualization on an ethidium bromide-stained agarose gel, along with molecular weight markers. The purified PCR products were radiolabeled by using a nick translation kit (Nick Translation System; Bethesda Research Laboratories, Gaithersburg, Md.) and [^{32}P]dCTP (specific activity, 650 Ci/mmol; ICN Biomedicals Inc., Irvine, Calif.) as previously described (41).

Dot blot hybridization assays were performed under high-stringency conditions as previously described (36). Briefly, nucleic acid samples were denatured at 100°C for 5 min and cooled on ice for 5 min. The samples were dotted onto 0.45-μm-pore-size nylon membranes (Zeta probe; Bio-Rad Laboratories, Richmond, Calif.) in volumes of 5 μl or less (0.1 to 0.5 μg) with 40 and 400 ng of RNA. Following application of the samples, the membranes were air dried and baked for 2 h at 80°C in a vacuum oven. The membranes were prehybridized for 4 h at 52°C in hybridization buffer (50% formamide, 5× standard saline citrate [SSC] [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate], 50 mM phosphate buffer [pH 6.5], 0.2% SDS, 2× Denhardt's solution, and 100 μg of yeast tRNA per ml). Hybridization was performed in 5 to 10 ml of hybridization buffer containing 3 × 10^6 to 5 × 10^6 cpm of heat-denaturated PCR probe (1.2 × 10^7 cpm/μg of DNA template). The hybridization was performed for 16 to 24 h at 52°C. The membranes were washed four times at room temperature in 2× SSC–0.1% SDS and two times at hybridization temperature in 0.4× SSC–0.1% SDS. The washed membranes were rinsed once with water, blotted, and exposed to Kodak XAR film with intensifying screens at −70°C for 5 to 7 days (36).

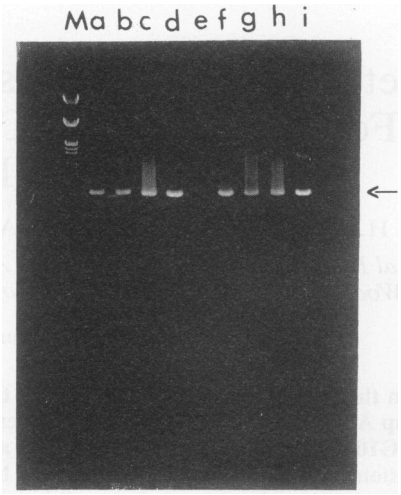

FIG. 1. Agarose gel electophoresis of the amplification products of the partial-length (341 bp) VP7 gene of rotavirus strains. Lanes: a, Wa (G1); b, DS-1 (G2); c, P (G3); d, Gottfried (G4); e, negative control; f, OSU (G5); g, 69M (G8); h, Cr (G10); i, YM (G11); M, molecular weight markers. The arrow indicates the partial-length PCR products.

The PCR VP7 partial-length cDNA fragments (nucleotides 51 to 392) of the VP7 gene of rotavirus serotypes G1 to G5, G8, G10, and G11 were generated by using the full-length cDNA copies of the VP7 gene from bovine, human, and porcine group A rotaviruses as templates. The amplified products from all the G types were similar in size (approximately 341 bp) (Fig. 1).

The dsRNA extracted from fecal samples was analyzed by PAGE to confirm the presence of viral dsRNA and to examine the genomic migration patterns (electropherotypes) of the BRV samples. The rotavirus-negative samples (controls) were confirmed by PAGE as negative for dsRNA (data not shown). Examples of the genome electropherotypes of some representative reference and field strains of BRV from our study are shown in Fig. 2.

The G-type specificities of the PCR-derived probes were tested by dot blot hybridization with dsRNA extracted from rotaviruses of several known serotypes of human and animal origin. Previous studies in our laboratory have established that in nucleic acid hybridization assays under high-stringency conditions (52°C, 50% formamide–5× SSC), the VP7 gene probes against serologically defined bovine (G6 and G10), human (G1, G2, G3, and G8), and porcine (G4, G5,

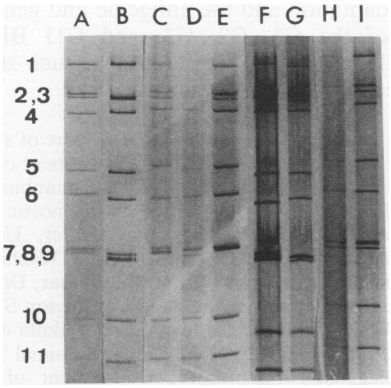

FIG. 2. Electrophoretic migration patterns of rotavirus dsRNA extracted from rotavirus-positive field specimens and some reference laboratory strains. Lanes A and B, NCDV and Cr strains, respectively; lanes C to I, field samples from Ohio (lanes C to G) and Nebraska (lanes H and I). The patterns shown are composites resolved in three separate polyacrylamide gels.

and G11) rotaviruses were G-serotype specific (36, 37, 39, 40). The PCR-generated probes did not cross-react with heterologous G-type bovine, human, and porcine rotaviruses.

In this study, 59 fecal samples from diarrheic calves positive for group A rotavirus by immune electron microscopy, ELISA, or PAGE were obtained. When analyzed for G6 and G10 serotypes by a monoclonal antibody-based serotyping ELISA (31) or by dot blot hybridization with the PCR-derived cDNA probes to BRV G6, G10, and human rotavirus G8 (69M strain) (37), all samples were negative, suggesting the presence of serotypes other than G6, G8, and G10.

In the present study, the PCR-derived cDNA probes to human and porcine rotaviruses were hybridized under high-stringency conditions with equal amounts of dsRNA extracted from the G6-, G8-, and G10-negative fecal samples from the diarrheic calves. The results are summarized in Table 1, and two representative blots are shown in Fig. 3 and 4. A total of seven samples hybridized with the G1-specific cDNA probe (the five positive samples from Ohio are shown in Fig. 3), one sample hybridized with the G2-specific cDNA probe, two samples hybridized with the G3 cDNA probe, and three additional samples hybridized with the G11 cDNA probe (the two positive samples from Ohio are shown in Fig. 4). One sample which tested positive with the G1 cDNA probe gave a weak hybridization signal with the G11 cDNA probe, and two samples positive with the G11 cDNA probe also gave weak hybridization signals with the G1 cDNA probe and the G1 and G3 cDNA probes, respectively (Table 1). Hybridization signals were not detected with control rotavirus-negative samples.

To date, at least four G serotypes (G1, G6, G8, and G10) have been reported to infect cattle (6, 44), with G6, G8, and G10 identified in the feces of diarrheic calves from the United States (37, 42). In a recent study in our laboratory, 102 field strains of BRV were characterized by using G6, G8, and G10 cDNA probes (37). The G-typing analysis revealed the presence of 37 G6, 3 G8, and 13 G10 BRV strains among samples obtained from diarrheic calves. The presence of G types other than G6 and G10 among BRV isolates was first reported by Snodgrass et al. (44), who demonstrated that BRV strains J2538 and 678, both from the United Kingdom, are serotype G8 (represented by human strain 69M) (32, 33, 44). The discovery of G8 serotypes of BRV among natural BRV strains was of added interest following a molecular

TABLE 1. Reactivity of BRV field samples with G-serotype-specific partial-length PCR-derived cDNA probes[a]

Origin	No. of samples (% positive)						
	Total	Positive for:				All G types	Untypeable
		G1	G2	G3	G11		
Ohio	35	5	1	1	2[b]	9 (25.7)	26 (74.3)
South Dakota	12	2[c]	0	0	1[d]	3 (25)	9 (75)
Nebraska	9	0	0	1	0	1 (11.1)	8 (88.9)
Washington	3	0	0	0	0	0 (0)	3 (100)
Total	59	7 (11.9)	1 (1.7)	2 (3.4)	3 (5.1)	13 (22.0)	46 (78)

[a] cDNA probes were prepared by PCR amplification of the hyperdivergent regions of the VP7 gene (nucleotides 51 to 392) from human (G1, G2, and G3) and porcine (G4, G5, and G11) rotaviruses. No samples hybridized with the cDNA probes to G4 (Gottfried) and G5 (OSU) porcine rotaviruses. The field samples included tested negative with G6, G8, and G10 cDNA probes.

[b] One sample exhibited weak hybridization signals with the G1 and G3 cDNA probes.

[c] One sample exhibited weak hybridization signals with the G11 cDNA probe.

[d] The sample exhibited weak hybridization signals with the G1 cDNA probe.

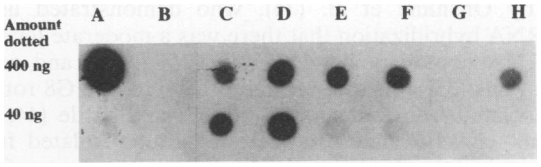

FIG. 3. Dot blot hybridization assay of BRV field samples with the human rotavirus strain Wa (G1) PCR-derived cDNA probe. The samples were probed under high-stringency conditions (52°C, 50% formamide–5× SSC). Lane A, positive control sample (Wa human rotavirus); lane B, negative control sample. With the G1 (Wa) cDNA probe under high-stringency conditions, hybridization signals were observed with five BRV field strains from Ohio (lanes C to F, and H). The sample in lane G from Ohio was negative.

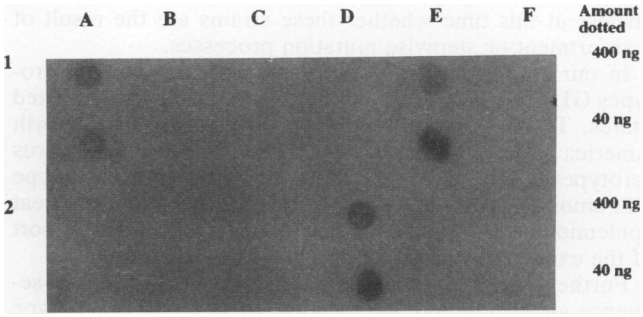

FIG. 4. Dot blot hybridization assay of BRV field samples with the porcine rotavirus strain YM (G11) PCR-derived cDNA probe. The samples were probed under high-stringency conditions (52°C, 50% formamide–5× SSC). Dot A1 is the positive control sample (G11 PCR product); B1 is the negative control sample. With the G11 (YM) cDNA probe under high-stringency conditions, hybridization signals were observed with two BRV field strains from Ohio (dots D2 and E1). No hybridization signals were observed with other samples from Ohio (dots A2, B1, B2, C1, C2, D1, E2, F1, and F2).

study by Ohshima et al. (34), who demonstrated using RNA-RNA hybridization that there was a moderate level of RNA-RNA homology between the 69M strain and some BRV strains (35, 44). Interestingly, the serotype G8 rotaviruses isolated from humans (1, 20, 33) and cattle (44, 49) were not closely related to the genogroups isolated from these species. Snodgrass et al. (45) reported that one of the serotype G8 rotavirus strains (J2538) possessed a typical BRV VP4 type P1, similar to that of the bovine isolate A5 from Thailand (49).

In addition to reporting G6, G8, and G10 BRV, several investigators have reported the presence of other G types among BRV (6, 7, 44). Among animals, pigs (5) and later calves (6, 7) were reported to have rotaviruses antigenically related to human rotavirus serotypes G1 and G2, as determined by their reactivity with monoclonal antibodies specific for human rotaviruses G1, G2, and G3 in a serotyping ELISA (6, 7). Recently, the BRV strain T449 was further characterized as serotype G1 by its one-way cross-reactivity in neutralization assays with antisera to the human G1 rotavirus (Wa strain) and by its deduced amino acid sequence homology (90% identity) with the human Wa rotavirus (6). The G11 porcine rotavirus strain YM was initially isolated from the feces of a diarrheic pig in Mexico (2). More recently, G11 rotavirus strains were also identified in our laboratory in the feces of diarrheic pigs from the United States by using cDNA probes derived from the VP7 gene of the Mexican G11 strain (39).

Our results have demonstrated that genetic diversity in terms of G serotypes exists among the BRV strains that we tested. In earlier studies, several investigators have suggested that previously undetected G types among rotaviruses might have arisen by genomic reassortment between human and animal rotaviruses (6, 11, 45, 49). Blackhall et al. (6) suggested that the VP7 gene of the T449 strain of BRV serotype G1 may have originated from reassortment between animal and human rotavirus strains rather than having undergone stepwise mutation to evolve as a serotype G1 rotavirus. In our study, the reaction of three samples with more than one serotype may be either due to the presence of multiple strains (two or three serotypes in the same specimen) or due to similarities at the genomic level between some of these newly identified BRV strains. Furthermore, the VP7 gene of the BRV strains which we have identified as G1, G2, G3, and G11 may have originated from a porcine or human source by genomic reassortment. However, it is unclear at this time whether these strains are the result of reassortment or stepwise mutation processes.

In our study, we have confirmed the existence of serotypes G1, G2, G3, and G11 among BRV strains in the United States. To our knowledge, this is the first report in North America of the identification among BRV strains of rotavirus serotypes G1, G2, G3, and G11. The discovery of serotype G11 among infected calves is of added interest and has great epidemiological significance because this is the only report of the existence of a G11 type among BRV strains.

Further antigenic and molecular analyses such as sequence determination are needed to confirm the G-type classification of the G1, G2, G3, and G11 BRV strains detected with the G-type-specific cDNA probes. Moreover, the origin, diversity, and distribution of rotavirus G serotypes occurring under field conditions in the bovine population needs to be addressed in additional studies in order to more closely monitor rotavirus strains circulating in animal populations, especially those viruses closely related to human strains. Future work in our laboratory will focus on the cell culture adaptation and the antigenic and genomic characterization of the G1, G2, G3, and G11 BRV strains identified in this study. It is possible that such strains have potential as candidates for human vaccines.

The samples from Ohio were submitted as part of the NAHMS study by Susan Lance and Paul Bartlett, Department of Veterinary Preventive Medicine, Ohio State University, Columbus, Ohio. The samples from Nebraska were submitted as diagnostic samples by Fernando Osorio, Veterinary Diagnostic Center, University of Nebraska, Lincoln, Nebr. The samples from Washington were submitted as diagnostic samples by Thomas Besser, Department of Veterinary Microbiology and Pathology, Washington State University, Pullman, Wash. The samples from South Dakota were submitted as diagnostic samples by D. H. Zeman, Animal Disease Research and Diagnostic Laboratory, Department of Veterinary Science, South Dakota State University, Brookings, S.Dak.

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