Detection of Group B Rotaviruses in Fecal Samples from Diarrheic Calves and Adult Cows and Characterization of Their VP7 Genes†

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Rotaviruses, members of the Reoviridae family, have a triple-layered capsid and a genome that consists of 11 segments of double-stranded RNA (dsRNA) (12, 15). Rotaviruses are divided into seven morphologically indistinguishable but antigenically distinct serogroups (12, 15). Group A rotaviruses are a common cause of diarrhea in calves (22, 25). Although non-group A (groups B and C) rotaviruses have been identified in cattle, there is little information on their prevalence (22, 25).

Group B rotaviruses may be emerging pathogens for humans and appear to be associated with diarrhea in adults and older children more than in neonates (3, 22). In calves and adult cows, group B rotaviruses have been reported in association with sporadic cases of diarrhea (1, 2, 18, 19). The group B rotaviruses are difficult to grow in cell culture, and in the host they are shed in low titers in the feces, making it difficult to detect and further characterize these viruses (3, 22).

Several assays to detect group B rotaviruses have been described. These include polyacrylamide gel electrophoresis (PAGE), immune electron microscopy (IEM), enzyme-linked immunosorbent assay (ELISA), cDNA hybridization, and reverse transcription-PCR (RT-PCR) (10, 11, 13, 30). Because of the limited shedding or instability of group B rotaviruses in the presence of pancreatin (50 μg/ml) (19, 22, 24, 29). All group A strains and the group C Shintoku strain were grown in Rhesus monkey kidney cells in the tissue culture system of Laemmli was utilized, and dsRNA was resolved in 10% polyacrylamide gel electrophoresis (PAGE), and reverse transcription (RT)-PCR (targeting 279 bp of the VP7 gene). In addition, 53 fecal samples from diarrheic adult cows were tested for group B rotaviruses by immune electron microscopy (IEM). By RT-PCR, five samples from calves were group B rotavirus positive (5.6%). Fifteen samples from adult cows with diarrhea were group B rotavirus positive (18.5%), and none of the control fecal samples from normal cows were positive for group B rotaviruses. By PAGE, one calf sample (RT-PCR positive) was group B rotavirus positive (short electropherotype), but none of the adult cow samples were positive for group B rotaviruses. By IEM, 5 (9.4%) of the 53 fecal samples from diarrheic adult cows were group B positive (all were also RT-PCR positive). The VP7 genes of three strains (WD653 from an adult cow and the ATI and Mebus calf strains) were sequenced. The VP7 genes from the three bovine strains showed high (over 90%) nucleotide and deduced amino acid homologies, but lower homologies (48 to 61%) were seen between these genes and the genes from rodent (IDIR) and human (ADRV) group B rotaviruses. Although there were some differences of degree, all inoculated gnotobiotic calves (n = 6) showed abnormal feces between 1 and 3 days after inoculation with each of three strains of group B bovine rotaviruses, and group B rotaviruses were detected in the feces for up to 2 weeks by RT-PCR but for shorter periods by PAGE or IEM.

MATERIALS AND METHODS

Virus and cells. The reference group A BRV strains have been described previously and include NCDV-Lincoln (G6P[1]), IND (G6P[5]), B23 (G10P[11]), and 2292B (G10P[11]) strains (4). The group B BRV strains include ATI and the Mebus strains (from calves) and Mebus strains from adult cows; the group C BRV include the Shintoku strain (19, 22, 24, 29). All group A strains and the group C Shintoku strain were grown in Rhesus monkey kidney cells in the presence of pancreatin (50 μg/ml) (21, 29). The group B rotaviruses, which were not cultivatable in cell culture, were amplified by passage in gnotobiotic calves, and infected intestinal contents were collected as a source of virus. As negative controls, mock-infected cell culture samples and mock-infected gnotobiotic calf feces were used.

Field samples. Analysis for group A and group B rotaviruses was performed on 90 fecal samples from diarrheic calves from Ohio, California, Wyoming, South Dakota, and Nebraska; 81 fecal samples from diarrheic adult cows (winter dysentery cases) from Ohio, New York, and California; and 20 fecal samples from normal adult cows from two Ohio herds.

Extraction and electrophoresis of dsRNA and IEM. Rotavirus dsRNA was extracted from cell culture-propagated viruses and fecal viruses by previously described procedures (14). Rotavirus dsRNA in extracted samples was analyzed by PAGE to confirm the presence of dsRNA and to examine the genomic electrophoretic types as previously described (4, 14). The discontinuous buffer system of Laemmli was utilized, and dsRNA was resolved in 10% polyacrylamide gel electrophoresis (PAGE) and reverse transcription (RT)-PCR (targeting 279 bp of the VP7 gene). In addition, 53 fecal samples from diarrheic adult cows were tested for group B rotaviruses by immune electron microscopy (IEM). By RT-PCR, five samples from calves were group B rotavirus positive (5.6%). Fifteen samples from adult cows with diarrhea were group B rotavirus positive (18.5%), and none of the control fecal samples from normal cows were positive for group B rotaviruses. By PAGE, one calf sample (RT-PCR positive) was group B rotavirus positive (short electropherotype), but none of the adult cow samples were positive for group B rotaviruses. By IEM, 5 (9.4%) of the 53 fecal samples from diarrheic adult cows were group B positive (all were also RT-PCR positive). The VP7 genes of three strains (WD653 from an adult cow and the ATI and Mebus calf strains) were sequenced. The VP7 genes from the three bovine strains showed high (over 90%) nucleotide and deduced amino acid homologies, but lower homologies (48 to 61%) were seen between these genes and the genes from rodent (IDIR) and human (ADRV) group B rotaviruses. Although there were some differences of degree, all inoculated gnotobiotic calves (n = 6) showed abnormal feces between 1 and 3 days after inoculation with each of three strains of group B bovine rotaviruses, and group B rotaviruses were detected in the feces for up to 2 weeks by RT-PCR but for shorter periods by PAGE or IEM.

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slab gels. Electrophoresis was conducted at 12 mA for 14 to 16 h. The dsRNA bands were visualized by silver staining (14). Fifty-three fecal samples from diarrheic adult cows were processed for IEM as previously described (19). The processed samples were incubated separately with group A-, B-, or C- rotavirus- or bovine coronavirus-specific hyperimmune antisera, and the mixtures were then pelleted, negatively stained with phosphotungstic acid, applied to grids, and examined by electron microscopy (19, 23).

RT-PCR assay. RT-PCR was used to produce full-length BRV VP7 (group A) and partial-length BRV VP7 (group B) cDNA fragments. cDNA was synthesized and amplified in an RT-PCR mixture containing 10× buffer (200 mM Tris-HCl [pH 8.3], 2.5 mM MgCl₂, 50 mM KCl, 0.05% gelatin), 10 mM (each) deoxyribonucleoside triphosphate, 200 ng of primer A (for group A, 5'-GGCCGGAT TTAAAAGCGACAATTT-3'; for group B, 5'-CAGTAACCTCTATCCTT TTACC-3'), 200 ng of primer B (for group A antisense, 5'-GGTACGATATCAACAATCTA-3'; for group B antisense, 5'-CGTATCGCAATACAATCCG-3') (4, 7, 8), 5 U of RNasin (Boehringer Mannheim Biochemicals, Indianapolis, Ind.), 10 U of avian myeloblastosis virus reverse transcriptase (Boehringer Mannheim Biochemicals), and 2.5 U of Taq polymerase (Boehringer Mannheim Biochemicals). First-strand cDNA synthesis was accomplished by incubating the above mixture for 90 min at 42°C. Thirty amplification cycles were conducted, with each cycle consisting of 94°C for 1 min (denaturation), 72°C for 1 min (annealing), and 72°C for 2 min (extension), followed by a 7-min extension at 72°C. For confirming group B rotavirus, nested PCR was performed, which produces a 239-bp product (see Fig. 2). The PCR products were analyzed on 1% agarose gels by using standard procedures (26).

Cloning and sequence analysis of the full-length VP7 genes of group B BRV. After determining the 5' and 3' end sequences by single primer amplification (16), the full-length VP7 genes of three group B rotaviruses (ATI and Mebus [calf] and WD653 [adult cow]) were produced by RT-PCR with 5' and 3' end primers and cloned in the pCR II vector (Invitrogen, San Diego, Calif.). The sequence analysis was performed using the primer extension method (Sequenase v2.0; Amersham, Arlington Heights, Ill.), and the data were analyzed using the DNASTAR program (DNASTAR Inc., Madison, Wis.).

Inoculation of gnotobiotic calves with group B rotaviruses. Three samples containing only group B BRV (determined by IEM and RT-PCR) were passaged in newborn (1- to 5-day old) gnotobiotic calves which had been derived, fed, inoculated, and maintained as previously described (19). All calves were oronasally inoculated with diluted (1:10) fecal filtrates of live virulent group B BRV strains (ATI and Mebus [calf] and WD653 [adult cow]). After inoculation, calves were examined twice daily for development of diarrhea, and the fecal consistency was scored (0, normal; 1, pasty; 2, semiliquid; 3, liquid). Each sample was tested for groups A and B rotaviruses by using PAGE, IEM and RT-PCR as described above.

Nucleotide sequence accession numbers. GenBank accession numbers for the VP7 genes of WD653, ATI, and Mebus are U844141, U844472, and U844473, respectively.

RESULTS

Electropherotypes. The group A and group B rotaviruses displayed typical electropherotypes (for group A, 4/2/3/2 dsRNA distribution patterns and for group B, 4/2/2/3 dsRNA patterns) (25) (Fig. 1). Like group A rotaviruses, group B rotaviruses showed short and long electropherotypes: the ATI and Mebus calf strains showed long patterns and the two adult strains, KD and WD653, showed short patterns (Fig. 1).

RT-PCR. As expected, the full-length VP7 gene (1,062 bp) was generated for group A rotaviruses and a partial-length VP7 gene was generated for group B rotaviruses (279 bp) (Fig. 2).

<table>
<thead>
<tr>
<th>State of sample origin</th>
<th>Total no. of samples</th>
<th>No. of group B rotavirus-positive samples by:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>RT-PCR</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Group A positive</td>
</tr>
<tr>
<td>Ohio</td>
<td>2/35</td>
<td>1/23</td>
</tr>
<tr>
<td>California</td>
<td>1/6</td>
<td>1/5</td>
</tr>
<tr>
<td>South Dakota</td>
<td>2/31</td>
<td>1/27</td>
</tr>
<tr>
<td>Wyoming</td>
<td>0/3</td>
<td>0/2</td>
</tr>
<tr>
<td>Nebraska</td>
<td>0/15</td>
<td>0/9</td>
</tr>
<tr>
<td>Total (%)</td>
<td>5/90 (5.6)</td>
<td>3/66</td>
</tr>
</tbody>
</table>

* Determined by RT-PCR.

One sample was group A rotavirus positive by PAGE and RT-PCR.
The RT-PCR assay was rotavirus group specific because there was no PCR product when the RT-PCR assay was applied to the heterologous group rotaviruses and negative control samples.

**Prevalence of group B BRV from field samples.** Based on our RT-PCR assay, 5 of 90 samples from diarrheic calves were group B BRV positive (5.6%); three of the five samples were also positive for group A BRV (Table 1). Fifteen samples from a total of 81 adult cows with diarrhea were group B rotavirus positive (18.5%) (Table 2). None of the fecal samples from normal case-matched control cows were positive for group A or group B BRV. By PAGE, only one calf sample (also RT-PCR positive, short electropherotype) was group B BRV positive, none of the adult cow samples were positive for B rotaviruses, and one adult cow sample (also RT-PCR positive) was positive for group A BRV (Table 2). By IEM, 29 (54.7%) of 53 samples from adult cows with diarrhea were coronavirus positive; 5 (9.4%) samples were group B positive (all were also group B positive by RT-PCR), 2 of which were also coronavirus positive; and 1 sample was group A positive.

**Sequence analysis of full-length VP7 genes of group B BRV.**

The full-length VP7 genes of group B BRV (ATI, Mebus, and WD653 strains) were PCR amplified, cloned, and sequenced. The full-length VP7 genes of the group B BRV were 811 bp in length, which was a little different from that of IDIR (804 bp; GenBank accession number, D00911) and ADRV (814 bp; GenBank accession number, M33872). The VP7 nucleotide similarities showed high homologies (over 90%) among the three bovine strains and greater diversities between the bovine strains and the IDIR or ADRV interspecies group B rotaviruses (about 51 to 57%) (Table 3). Also, the deduced amino acid identities were high among bovine strains but were lower between the bovine strains and the IDIR or ADRV interspecies group B rotaviruses (about 48 to 61%) (Table 3).

**Pathogenicity and virus-shedding patterns of group B BRV in gnotobiotic calves.** Although there were some differences of degree, all inoculated calves showed abnormal feces between 1 and 3 days after inoculation. The viruses were detected in the feces by IEM and PAGE for 1 to 6 days postinoculation and for up to 2 weeks postinoculation by RT-PCR (Table 4).

**DISCUSSION**

The group B rotaviruses have been regarded as an emerging pathogen for humans and appear to be associated more with older ages than with infants (3, 22). The group B rotaviruses have also been reported among mice, pigs, sheep, and cattle (2, 3, 19, 28). Although there is one report of adapting a porcine group B rotavirus to cell culture (27), the growth of group B rotaviruses in a continuous cell line has not been accomplished. Group B BRV are fastidious in their growth requirements, and this, coupled with the fact that these viruses are shed in low quantities in the feces of infected animals, has hampered their further characterization.

Group B rotaviruses have been identified by serology and dsRNA electrophoresis (3, 22, 24). In addition, there is a characteristic production of enterocyte syncytia for group B rotaviruses (3, 18, 22, 24). Group B BRV have been identified in the United States and the United Kingdom (2, 3, 7, 8, 18, 19, 22, 24), but information on their prevalence is very limited. In a serological study in the United Kingdom, 71% of adult cow sera were positive to human ADRV strain (2), and in another study, 20% of cow sera were positive to porcine NIRD-1 strain (2). In the United States, Chinsangaram et al. (7) reported that 81% (38 of 47) of dairy calves (1 to 14 days old) in one calf ranch and in one dairy herd were group B positive by RT-PCR but were not positive by PAGE. However, in our study, using identical primers, the prevalence of group B rotaviruses in feces from diarrheic calves from five states was only 5.6% (5 of 90), and interestingly, in feces from diarrheic adult cows (winter dysentery samples), the prevalence was 18.5% (15 of 81). Of the samples from diarrheic calves, three samples (out of five) were both group A and group B positive by RT-PCR, which suggests that mixed group A and group B rotavirus infections in calves might be common in the field. By PAGE, only one sample (1 of 171) from calves was group B positive (short electropherotype), which supports the hypothesis that

### Table 3. Nucleotide and deduced amino acid sequence comparisons of the VP7 genes of bovine (ATI, Mebus, and WD653), human (ADVR), and rat (IDIR) group B rotaviruses

<table>
<thead>
<tr>
<th>Strain</th>
<th>ATI</th>
<th>Mebus</th>
<th>WD653</th>
<th>ADRV</th>
<th>IDIR</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ATI</td>
<td>95.2</td>
<td>91.3</td>
<td>56.8</td>
<td>50.8</td>
<td></td>
</tr>
<tr>
<td>Mebus</td>
<td>96.8</td>
<td>89.5</td>
<td>56.5</td>
<td>51.5</td>
<td></td>
</tr>
<tr>
<td>WD653</td>
<td>92.7</td>
<td>92.3</td>
<td>55.2</td>
<td>51.1</td>
<td></td>
</tr>
<tr>
<td>ADRV</td>
<td>59.7</td>
<td>59.3</td>
<td>61.3</td>
<td></td>
<td>50.8</td>
</tr>
<tr>
<td>IDIR</td>
<td>48.6</td>
<td>48.2</td>
<td>48.6</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Data above the diagonal are nucleotide sequence similarities; data below the diagonal are amino acid sequence similarities. Analysis was performed by using the DNASTAR program.
* GenBank accession number, M33872.
* GenBank accession number, U84473.
* GenBank accession number, U84472.
* GenBank accession number, M33872.
* GenBank accession number, D00911.

### Table 4. Pathogenicity of the ATI, Mebus, and WD653 strains of group B BRV in gnotobiotic calves

<table>
<thead>
<tr>
<th>Strain</th>
<th>Diarrhea/virus shedding at PID:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>ATI</td>
<td>−/−</td>
</tr>
<tr>
<td>Mebus</td>
<td>−/+</td>
</tr>
<tr>
<td>WD653</td>
<td>−/+</td>
</tr>
<tr>
<td>A</td>
<td>−/+</td>
</tr>
<tr>
<td>B</td>
<td>−/+</td>
</tr>
<tr>
<td>C</td>
<td>−/+</td>
</tr>
<tr>
<td>D</td>
<td>−/+</td>
</tr>
</tbody>
</table>

* Calves were 1 to 5 days old at the time of inoculation. Presence or absence of diarrhea and virus shedding (determined by RT-PCR) indicated by plus or minus signs, respectively. PID, postinoculation day.
* ND, not done.
* Four calves (A, B, C, and D) were inoculated with WD653 strain.
group B rotavirus shedding is low in the infected hosts. The discrepancy between our results and those of the study by Chinsangaram et al. (7) could be attributed to geographical differences, sporadic outbreak of group B infections on farms, or differences in timing of sample collection. Our results indicate that group B rotaviruses may play a role in sporadic cases of calf and adult cow diarrhea in the field and may be more associated with adult cattle diarrhea. In addition, although bovine coronavirus may play a major role in winter dysentery in cows (24), other enteric pathogens including bovine group B rotaviruses may also be involved in this syndrome.

The genetic variation which exists among group B rotaviruses from different host species is poorly characterized, but based on analysis of nucleotide and deduced amino acid sequence data, group B rotaviruses show higher diversity than group A or group C rotaviruses (6, 9, 20). For molecular comparisons of intraspecies and interspecies group B rotaviruses, we sequenced the full-length VP7 genes of three field strains of group B BRV (ATI, Mebus, and WD653) and compared them to the VP7 gene sequences of two heterologous group B rotaviruses, ADRV from humans and IDIR from rats. The similarity of the VP7 gene nucleotide sequences between bovine strains and the heterologous group B rotaviruses was about 51 to 57%, and the amino acid homologies ranged from 48 to 61%. For group A rotaviruses, the homology among different G types (VP7 serotypes) is about 70 to 80% at the nucleotide level and about 60% at the amino acid sequence level (17). Regardless of their origin (adult cows or calves) and of their electropherotype (short or long), all three bovine strains showed high homologies in their VP7 genes: over 90% in nucleotide and amino acid sequences. However, when group B rotavirus strains from heterologous species, i.e., humans and rats, were included, the VP7 genes showed high diversity. The reason for this high diversity among the VP7 genes of group B rotaviruses from different hosts is not clear and requires further study.

After inoculating three group B BRV strains (ATI, Mebus, and WD653) into gnotobiotic calves, all three strains induced at least mild diarrhea, but the clinical signs and the duration of virus shedding varied among calves even with the same strain. Such variability might be due to the timing of inoculation (age dependence), virus instability, or the different viral concentrations of each inoculum, all of which were low in comparison with those of group A rotavirus inocula. Additional studies are underway to confirm the pathogenicity of and to further characterize additional group B BRV strains.

ACKNOWLEDGMENTS

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