Identification and Molecular Characterization of a Bovine G3 Rotavirus Which Causes Age-Independent Diarrhea in Cattle

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G3 rotaviruses have been reported rarely in cattle, and none have been characterized. We report the first genomic characterization of a bovine G3 rotavirus, CP-1, which had been biologically characterized in vivo and shown to cause age-independent diarrhea. CP-1 was a G3 rotavirus as its VP7 had 92 to 96% deduced amino acid identity to those of G3 rotaviruses. However, initially, CP-1 was identified as a G10 rotavirus by RT-PCR even though the CP-1 VP7 had only 81 to 85% deduced amino acid identity to those of G10 rotaviruses. VP7 gene sequencing, viral RNA was extracted from cell culture fluids by phenol-chloroform or by the method of Boom et al. (3). The G-typing RT-PCR was conducted as described by Gouvea et al. (16) with the G5, G6, G8, and G10 primers but using the Expand High Fidelity PCR System (Roche). Rotaviruses OSU, UK, 678, and B225 were used as reference G5, G6, G8, and G10 rotaviruses, respectively. A G5-G6-G8-G10 primer pool or individual G-type-specific primers were used. Amplicons were electrophoresed on 2% agarose gels and visualized by short wave UV light after staining with ethidium bromide. For VP4 gene sequencing, a VP4 cDNA fragment of 811 nucleotides encoding VP4*, the connecting peptide, and the termini of VP5* was produced by RT-PCR using forward (EBH1, 5'-GTTACCGGCAGCAGCGGCTATAAATTGAC-3') and reverse (RVA4-1, 5'-GGTCACATTTTATGAT-3') primers which were based on nucleotides 1 to 19 and nucleotides 793 to 811 (underlined) of the bovine rotavirus UK VP4 gene plus added cloning sites. The CDNA was cloned into pGEM-3Z (Promega Corp.) after digestion with PvuI and BamHI restriction enzymes, and the plasmid was transfected into competent XL1-Blue E. coli cells (Strategene). Two or three gene clones from two PCR products were sequenced in both forward and reverse directions. The CP-1 VP7 and NSP4 genes were reverse transcribed using hexamer primers. The VP7 gene was amplified using the forward and reverse gene-specific primers Bg9, End9, End9 UK, and CRW-8 (15). The NSP4 gene was amplified using the reverse primer 5'-CAT(A/C)(G)(A/C)(T/G)CA G(T/C)ACTCT-3' and a pool of two forward primers: 5'-ATGGAAAAAGTTT CCGACCTC-3' and 5'-GGCTTTTTAAAGTCTGTTT-3' (modified from reference 25). VP7 and NSP4 amplicons were cloned into pCR2.1 vector and using a TOPO TA cloning kit (Invitrogen) as described by the manufacturer, and two clones were sequenced in both directions using standard M13 forward and reverse primers (5'-GTAACACGCGCGGAGGC-3' and 5'-CAGGAACACGCT ATGAC-3'). The CP-1 NSP1 gene was reverse transcribed using a gene-specific primer representing the 3' end of NSP1 (5'-GGTACCATTTTAT GCTGCCTA-3') and amplified using the same primer and a forward primer representing the 5' end (5'-GGCCCGTTTTTTATGA-3') (modified from reference 30). NSP1 amplicons were sequenced directly from PCR products after gel purification using a Qiagen Quick kit. Sequencing was conducted with an ALF automated sequencer (Pharmacia) or an ABI 3700 sequencing system.
(MWG-Biotech). Consensus sequences were compared using the DNAstar or the NCBI BLAST sequence analysis programs. Multiple sequence alignments were performed using either the NCBI or Clustal X or EBI Clustal W programs. Phylogenetic analyses were conducted using the Clustal W neighbor-joining method. The VP7 monoclonal antibodies B223-N7 and B223/3 raised against the G10P[11] bovine rotavirus B223 were supplied by D. Snodgrass and G. N. Wood. MA104 cells were infected overnight, and G10P[11] bovine rotavirus B223 were supplied by D. Snodgrass and G. N. Wood. MA104 cells were infected overnight, and stained with monoclonal antibodies at a 1:500 dilution, followed by a 1:200 dilution of peroxidase-conjugated rabbit anti-mouse immunoglobulin (DAKO). Color was developed with 3,3-diaminobenzidine tetrahydrochloride tablets (Kem-En-Tec, Copenhagen, Denmark).

The VP4 gene sequences used were as follows (accession numbers are given in parentheses): C486 (X00127), UK (M22306), B223 (M92986), 99983 (D16352), B641 (P25173), KK3 (P36308), A44 (P36307), 61A (P36306), A5 (P36305), and NCDV (P17465). The VP7 gene sequences used were as follows (accession numbers are given in parentheses): T449 (M92651), CRW-8 (edited from reference 20), AU-1 (D96271), SA11 (X6158), UK (X00896), 99383 (X08669), 678 (L20883), B223 (X57052), P343 (edited from reference 28), B60 (M64880), A44 (D01055), B11 (M64679), 61A (X53403), 2292B (U14996) KK3 (D01056), EW (U08430), 97-848 (AF260957), 98-B31 (AF260958), MP126 (AF386915), 02/92 (D01055), B11 (M64679), 61A (X53403), 2292B (U14996) KK3 (D01056), EW (U08430), 97-848 (AF260957), 98-B31 (AF260958), MP126 (AF386915), 02/92 (D01055). The NSP1 gene sequences used were as follows (accession numbers are given in parentheses): UKtc (Z12108), RF (M22308), A44 (U23726), OSU (U08432), Gottfried (U08431), YM (D38154), Wa (L18943), DS1 (L18945), and H2 (D38157). The NSP4 gene sequences used were as follows (accession numbers are given in parentheses): UK (K03384), NCDV (X06086), B223 (AF144805), BRV033 (AF144804), OSU (D88831), A253(144797), YM (X69485), A131 (AF144798), A41 (AF144799), Wa (AF093199), AU32 (D88830), KUN (D88829), ST3 (US9110), M37 (US9110), RV4 (US9108), 1076 (US9105), AU-1 (D89873), VA70 (U38798), RV5 (US9103), FRV64 (D88833), CU-1 (AF144806), EW (U96335), EHP (U96336), and EC (U96337). Accession numbers for the following rotavirus CP-1 genes are as indicated: VP4, AF448851; VP7, AF448852; NSP1, AF448853; and NSP4, AF448854.

**RESULTS AND DISCUSSION**

CP-1 had a typical group A rotavirus genome profile on 10% polyacrylamide gels (Fig. 1). Gene segments 1 to 3, 5, 6, 10, and 11 migrated indistinguishably from the equivalent segments of the G6P[5] rotavirus UK run in the parallel track. There were differences between the two rotaviruses in the 7–8–9 triplet region and in the 3–4 region, where CP-1 showed a slightly bigger gap between segments 3 and 4 than UK. By RT-PCR using the published ET10 primer for G10 rotaviruses (16), CP-1 was initially typed as a G10 rotavirus (Fig. 2). The expected 715-bp amplicon was produced with CP-1, the reference G10 bovine rotavirus B223, the recently characterized human G3P[8] rotavirus 200/97, and the recently characterized G3P[7] bovine-porcine reassortant PP-1 (11). However, immunoperoxidase tests with the G10-specific monoclonal antibodies B223/3 and B223-N7 failed to confirm G10 specificity for CP-1. Previously, sequence analysis of the ET10 amplicon of the bovine-porcine reassortant PP-1 showed G3, not G10, specificity: the amplicon had 93% deduced amino acid identity to G3 rotaviruses and 77% identity to G10 rotaviruses (11). The initial misdiagnosis of CP-1 and PP-1 as G10 rotaviruses by PCR was explained by the similarity of the CP-1 and PP-1 nucleotide sequences to the ET10 primer in the primer binding region, nucleotides 697 and 712 (Fig. 3). Both viruses differed from the ET10 G10 primer by only two nucleotides located in the middle of the G10 primer. Furthermore, the human G3P[8] rotavirus 200/97, which produced an amplicon with the ET10 primer of the size expected for G10 rotaviruses in our hands, had an identical or almost-identical (one nucleotide was undetermined) composition to CP-1 and PP-1 in the primer binding region. At least five additional human G3 rotaviruses (Hu/97-S48, Hu/98-B31, Hu/MP126, Hu/02/92, and Hu/107elb) also have nucleotide sequences identical to CP-1 and PP-1 in this region. Furthermore, the established G3 rotavirus SA11 has the same two differences as CP-1 plus one other.

Sequence analysis of the full-length CP-1 VP7 showed it to be a G3, not G10, rotavirus with 92 to 96% deduced amino acid (81 to 90% nucleotide) identity to the porcine G3 rotavirus CRW-8, the human G3 rotavirus AU-1, and the simian G3 rotavirus SA11 and only 82 to 85% deduced amino acid (75 to 77% nucleotide) identity to the bovine G10 rotaviruses B223, 61A, KK3, A44, 2292B, and B11. The CP-1 VP7 had 57 to 83% amino acid (65 to 76% nucleotide) identity to the other G types found in cattle, the G1 rotavirus T449, the G6 rotaviruses UK and B60, the G7 rotavirus 995/83, and the G8 rotavirus 678. Phylogenetic analysis confirmed the grouping of CP-1 with G3 and not G10 rotaviruses (Fig. 4). Eight cysteine residues
were identified in the CP-1 VP7 protein, in common with other rotaviruses (Fig. 5). Eleven proline residues were identified. Nine were common to, and at the same positions as, the G3 rotaviruses AU-1, CRW-8, EW, and SA11; the G10 rotaviruses B223, B11, 61A, KK3, and A44; and the G10 rotaviruses B223, B11, 61A, KK3, and A44. Accession numbers are given in the text.

Sequence analysis of 267 deduced amino acids at the amino terminus of the CP-1 VP4 (representing VP8*, the interconnecting peptide and the 5' terminus of VP9*) identified CP-1 as a P[5] rotavirus with 92 to 99% deduced amino acid (91 to 99.5% nucleotide) identity to the bovine rotavirus UK and 92% amino acid (87% nucleotide) identity to the bovine rotaviruses RF and A44. It had less than 70% amino acid (72% nucleotide) identity to the porcine rotaviruses OSU, Gottfried, and YM; the human rotaviruses Wa and DS1; and the equine rotavirus H2 (data not shown)—thus endorsing previous findings that bovine rotavirus NSP1s segregate into a distinct group (27, 32).

Sequence analysis of the full-length CP-1 NSP4 also showed it to be almost identical to that of the bovine rotavirus UK, differing by two nucleotides but with an identical amino acid composition. It had 92 to 96% deduced amino acid (80 to 88% nucleotide) identity to the NSP4s of bovine rotaviruses NCDV, B223, and BRV033 but 83 to 86% deduced amino acid (77 to 80% nucleotide) identity to the porcine rotaviruses OSU, A253, YM, A131, and A411; 82 to 85% deduced amino acid (78 to 82% nucleotide) identity to the human rotaviruses Wa, AU32, M37, and AU-1; and 63 to 64% deduced amino acid (62 to 63% nucleotide) identity to the murine rotaviruses EW, EC, and EHP. The CP-1 NSP4 clustered with other bovine rotaviruses into genotype A by phylogenetic analysis (Fig. 6) (8). In the enterotoxin peptide region (amino acids 114 to 135) which was associated with age-dependent diarrhea in mice (2), CP-1, which caused age-independent diarrhea in cattle (6), differed by only one amino acid at position 135, with a mutation of threonine to methionine (data not shown). CP-1 was identical to the bovine rotavirus UK in the variable region.

FIG. 3. Comparison of the VP7 nucleotide sequence of the ET10 G10 primer (reverse complement); the G3P[5] bovine rotavirus CP-1; the G3P[7] bovine-porcine reassortant rotavirus PP-1; the G3 human rotaviruses 200/97, 97-S48, 98-B31, MP126, 02/92, 107eb, and AU-1; the G3 simian rotavirus SA11; and the G10 bovine rotaviruses B223, B11, 61A, KK3, and A44. Accession numbers are given in the text.

FIG. 4. Phylogenetic tree of VP7 deduced amino acid sequences of rotaviruses of G types 1, 3, 6, 8, and 10 and CP-1 produced by the Clustal W neighbor-joining method.
spanning amino acids 130 to 141 but differed from the bovine rotaviruses NCDV, B223, and BRV033 by three to six residues. Thus, rotavirus CP-1 was characterized as a G3P[5] rotavirus with a bovine NSP1 and NSP4. The high sequence identities of the CP-1 VP4, NSP1, and NSP4 to that of the G6P[5] bovine rotavirus UK were consistent with CP-1 and UK being isolated from the same fecal sample, albeit by different passage methods (4). These similarities were supported by the polyacrylamide gel electrophoresis genome analysis. The occasional G3 rotavirus has been found in cattle previously using PCR or PCR-generated cDNA probes (10, 22) but the present study is the first to report a bovine G3 VP7 sequence and the first to characterize the VP4, VP7, NSP1, and NSP4 of a bovine rotavirus which has been characterized experimentally in vivo (6, 17). The present study raises the possibility that G3 rotaviruses may be more common in cattle than currently thought, as CP-1.
was initially misdiagnosed as a G10 rotavirus by RT-PCR. In surveys in which G10 bovine rotaviruses have been found by PCR, G10 specificity has rarely been confirmed by sequencing (10, 12, 14, 21, 23). Misdiagnosis by PCR has been recorded previously, for example between G3 and G8 rotaviruses (1). Clearly, more sequence information is required from G3, G8, and G10 bovine rotaviruses from diverse locations so that primer specificity can be improved.

The finding that CP-1 had P[5] specificity and a bovine NSP1 and NSP4 was unremarkable except for showing that these three proteins can form viable infectious virions with the G3 VP7 protein and that these virions are capable of causing age-independent diarrhea in experimental calves (6), producing villous atrophy and malabsorption (17). It is interesting that CP-1 differed by only one amino acid from the NSP4 enterotoxin peptide which caused age-dependent diarrhea in cattle that CP-1 differed by only one amino acid from the NSP4.

ACKNOWLEDGMENTS

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REFERENCES


24. Iturriza-Gómez, M., J. Green, D. Brown, M. Ramsay, U. Desselberger, and NSP4 type with the ability to infect and cause age-dependent or age-independent diarrhea in cattle.


